BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

REGULATORY SEQUENCES FOR TRANSGENIC PLANTS

Related Applications

Field of the Invention

This invention relates to genetic engineering of plants. More particularly, the invention provides DNA sequences and constructs that are useful to control expression of recombinant genes in plants. Specific constructs of the invention use novel regulatory sequences derived from a maize root preferential cationic peroxidase gene.

Background of the Invention

Through the use of recombinant DNA technology and genetic engineering, it has become possible to introduce desired DNA sequences into plant cells to allow for the expression of proteins of interest. However, obtaining desired levels of expression remains a challenge. To express agronomically important genes in crops at desired levels through genetic engineering requires the ability to control the regulatory mechanisms governing expression in plants, and this requires access to suitable regulatory sequences that can be coupled with the genes it is desired to express.

A given project may require use of several different expression elements, for example one set to drive a selectable marker or reporter gene and another to drive the gene of interest. The selectable marker may not require the same expression level or pattern as that required for the gene of interest. Depending upon the particular project, there may be a need for constitutive expression, which directs transcription in most or all tissues at all times, or there may be a need for tissue specific expression. For example, a root specific or root preferential expression in maize would be highly desirable for use in expressing a protein toxic to pests that attack the roots of maize.

Cells use a number of regulatory mechanisms to control which genes are expressed and the level at which they are expressed. Regulation can be transcriptional or post-transcriptional and can include, for example, mechanisms to enhance, limit, or prevent transcription of the DNA, as well as mechanisms that limit the life span of the mRNA after it is produced. The DNA sequences involved in these regulatory processes can be located upstream, downstream or even internally to the structural DNA sequences encoding the protein product of a gene.

the transcriptional activation that has been described by many as constitutive. The 35S promoter is very efficiently expressed in most dicots and is moderately expressed in monocots. The addition of enhancer elements to this promoter has increased expression levels in maize and other monocots. Constitutive promoters of monocot origin (that are not as well studied) include the polyubiquitin-1 promoter and the rice actin-1 promoter. Wilmink *et al.* (1995). In addition, a recombinant promoter, Emu, has been constructed and shown to drive expression in monocots in a constitutive manner. Wilmink *et al.* (1995).

Few tissue specific promoters have been characterized in maize. The promoters from the zein gene and oleosin gene have been found to regulate GUS in a tissue specific manner. Kriz *et al.* (1987); Lee and Huang (1994). No root specific promoters from maize have been described in the literature. However, promoters of this type have been characterized in other plant species.

Despite both the important role of tissue specific promoters in plant development, and the opportunity that availability of a root preferential promoter would represent for plant biotechnology, relatively little work has yet been done on the regulation of gene expression in roots. Yamamoto reported the expression of *E. coli*: *uidA* gene, encoding β-glucuronidase (GUS), under control of the promoter of a tobacco (*N. tabacum*) root-specific gene, TobRB7. Yamamoto *et al.* (1991), Conkling *et al.* (1990). Root specific expression of the fusion genes was analyzed in transgenic tobacco. Significant expression was found in the root-tip meristem and vascular bundle. EPO Application Number 452 269 (De Framond) teaches that promoters from metallathionein-like genes are able to function as promoters of tissue-preferential transcription of associated DNA sequences in plants, particularly in the roots. Specifically, a promoter from a metallathionein-like gene was operably linked to a GUS reporter gene and tobacco leaf disks were transformed. The promoter was shown to express in roots, leaves and stems. WO 9113992 (Croy, *et al.*) teaches that rape (*Brassica napus* L.) extensin gene promoters are capable of directing tissue-preferential transcription of associated DNA sequences in plants, particularly in the roots. Specifically, a rape extensin gene promoter was operably linked to a *extA* (extensin structural gene) and tobacco leaf disks were transformed. It was reported that northern analysis revealed no hybridization of an extensin probe to leaf RNA from either control or transformed tobacco plants and hybridization of the extensin probe to transgenic root RNA of all transformants tested, although the levels of hybridization varied for the transformants tested. While each of these

promoters has shown some level of tissue-preferential gene expression in a dicot model system (tobacco), the specificity of these promoters, and expression patterns and levels resulting from activity of the promoters, has yet to be achieved in monocots, particularly maize.

DNA sequences called enhancer sequences have been identified which have been shown to enhance gene expression when placed proximal to the promoter. Such sequences have been identified from viral, bacterial, and plant gene sources. An example of a well characterized enhancer sequence is the *ocs* sequence from the octopine synthase gene in *Agrobacterium tumefaciens*. This short (40 bp) sequence has been shown to increase gene expression in both dicots and monocots, including maize, by significant levels. Tandem repeats of this enhancer have been shown to increase expression of the GUS gene eight-fold in maize. It remains unclear how these enhancer sequences function. Presumably enhancers bind activator proteins and thereby facilitate the binding of RNA polymerase II to the TATA box. Grunstein (1992). WO95/14098 describes testing of various multiple combinations of the *ocs* enhancer and the *mas* (mannopine synthase) enhancer which resulted in several hundred fold increase in gene expression of the GUS gene in transgenic tobacco callus.

The 5' untranslated leader sequence of mRNA, introns, and the 3' untranslated region of mRNA affect expression by their effect on post-transcription events, for example by facilitating translation or stabilizing mRNA.

Expression of heterologous plant genes has also been improved by optimization of the non-translated leader sequence, i.e. the 5' end of the mRNA extending from the 5' CAP site to the AUG translation initiation codon of the mRNA. The leader plays a critical role in translation initiation and in regulation of gene expression. For most eukaryotic mRNAs, translation initiates with the binding of the CAP binding protein to the mRNA CAP. This is then followed by the binding of several other translation factors, as well as the 43S ribosome pre-initiation complex. This complex travels down the mRNA molecule while scanning for an AUG initiation codon in an appropriate sequence context. Once this has been found, and with the addition of the 60S ribosomal subunit, the complete 80S initiation complex initiates protein translation. Pain (1986); Kozak (1986). Optimization of the leader sequence for binding to the ribosome complex has been shown to increase gene expression as a direct result of improved translation initiation efficiency. Significant increases in gene expression have been produced by addition of leader sequences from plant viruses or heat shock genes.

Raju *et al.* (1993); Austin (1994) reported that the length of the 5' non-translated leader was important for gene expression in protoplasts.

In addition to the untranslated leader sequence, the region directly around the AUG start appears to play an important role in translation initiation. Luerhsen and Walbot (1994). Optimization of the 9 bases around the AUG start site to a Kozak consensus sequence was reported to improve transient gene expression 10-fold in BMS protoplasts. McElroy *et al.* (1994).

Studies characterizing the role of introns in the regulation of gene expression have shown that the first intron of the maize alcohol dehydrogenase gene (*Adh-1*) has the ability to increase expression under anaerobiosis. Callis *et al.* (1987). The intron also stimulates expression (to a lesser degree) in the absence of anaerobiosis. This enhancement is thought to be a result of a stabilization of the pre-mRNA in the nucleus. Mascarenhas *et al.* reported a 12-fold and 20-fold enhancement of CAT expression by use of the *Adh-1* intron. Mascarenhas *et al.* (1990). Several other introns have been identified from maize and other monocots which increase gene expression. Vain *et al.* (1996).

The 3' end of the mRNA can also have a large effect on expression, and is believed to interact with the 5' CAP. Sullivan (1993). The 3'untranslated region (3'UTR) has been shown to have a significant role in gene expression of several maize genes. Specifically, a 200 base pair 3' sequence has been shown to be responsible for suppression of light induction of the maize small m3 subunit of the ribulose-1,5-biphosphate carboxylase gene (rbc/m3) in mesophyll cells. Viret *et al.* (1994). Some 3' UTRs have been shown to contain elements that appear to be involved in instability of the transcript. Sullivan *et al.* (1993). The 3'UTRs of most eukaryotic genes contain consensus sequences for polyadenylation. In plants, especially maize, this sequence is not very well conserved. The 3' untranslated region, including a polyadenylation signal, derived from a nopaline synthase gene (3' *nos*) is frequently used in plant genetic engineering. Few examples of heterologous 3'UTR testing in maize have been published.

Important aspects of the present invention are based on the discovery that DNA sequences derived from a maize root specific cationic peroxidase gene are exceptionally useful for use in regulating expression of recombinant genes in plants.

The peroxidases (donor:hydrogen-peroxide oxidoreductase, EC 1.11.1.7) are highly catalytic enzymes with many potential substrates in the plant. See Gaspar, *et al.* (1982).

They have been implicated in such diverse functions as secondary cell wall biosynthesis, wound-healing, auxin catabolism, and defense of plants against pathogen attack. See Lagrimini and Rothstein (1987); Morgens *et al.* (1990); Nakamura *et al.* (1988); Fujiyama *et al.* (1988); and Mazza *et al.* (1980).

Most higher plants possess a number of different peroxidase isozymes whose pattern of expression is tissue specific, developmentally regulated, and influenced by environmental factors. Lagrimini & Rothstein (1987). Based upon their isoelectric point, plant peroxidases are subdivided into three subgroups: anionic, moderately anionic, and cationic.

The function of anionic peroxidase isozymes (pI, 3.5-4.0) is best understood. Isozymes from this group are usually cell wall associated. They display a high activity for polymerization of cinnamyl alcohols *in vitro* and have been shown to function in lignification and cross-linking of extensin monomers and feruloylated polysaccharides. Lagrimini and Rothstein (1987). In both potato and tomato, expression of anionic peroxidases have been shown to be induced upon both wound induction and abscisic acid treatment. Buffard *et al.* (1990). This suggests their involvement in both wound healing and in the regulation of tissue suberization.

Moderately anionic peroxidase isozymes (pI, 4.5-6.5) are also cell wall associated and have some activity toward lignin precursors. In tobacco, isozymes of this class have been shown to be highly expressed in wounded stem tissue Fujiyama *et al.* (1988). These isozymes may also serve a function in suberization and wound healing. Morgens *et al.* (1990).

The actual function of cationic peroxidase isozymes (pI, 8.1-11) in the plant remains unclear. Some members of this group, however, have been shown to efficiently catalyze the synthesis of H_2O_2 from NADH and H_2O. Others are localized to the central vacuole. In the absence of H_2O_2, some of these isozymes possess indoleacetic acid oxidase activity. Lagrimini and Rothstein (1987).

Electrophoretic studies of maize peroxidases have revealed 13 major isozymes. Brewbaker *et al.* (1985). All isozymes were judged to be functional as monomers, despite major differences in molecular weight. All maize tissues had more than one active peroxidase locus, and all loci were tissue-specific. The peroxidases have proved unique in that no maize tissue has been found without activity, and no peroxidase has proven expressed in all maize tissues.

Summary Of The Invention

The invention provides isolated DNA molecules derived from the *per5* maize root preferential cationic peroxidase gene that can be used in recombinant constructs to control expression of genes in plants. More particularly, the invention provides isolated DNA molecules derived from the *per5* promoter sequence and having as at least a part of its sequence bp 4086-4148 of SEQ ID NO 1. Preferred embodiments are isolated DNA molecules that have as part of their sequences bp 4086 to 4200, bp 4086 to 4215, bp 3187 to 4148, bp 3187 to 4200, bp 3187 to 4215, bp 2532-4148, bp 2532 to 4200, bp 2532 to 4215, bp 1-4148, bp, bp 1-4200, or bp 1-4215 of SEQ ID NO 1.

The invention also provides isolated DNA molecules selected from the following *per5* intron sequences: bp 4426-5058 , bp 4420-5064, bp 5251-5382, bp 5245-5388, bp 5549-5649, and bp 5542-5654 of SEQ ID NO 1.

The invention also provides isolated DNA molecules derived from the *per5* transcription termination sequence and having the sequence of bp 6068-6431 of SEQ ID NO 1.

In another of its aspects, the present invention provides a recombinant gene cassette competent for effecting preferential expression of a gene of interest in a selected tissue of transformed maize, said gene cassette comprising:

a) a promoter from a first maize gene, said first maize gene being one that is naturally expressed preferentially in the selected tissue;

b) an untranslated leader sequence;

c) the gene of interest, said gene being one other than said first maize gene;

d) a 3'UTR;

said promoter, untranslated sequence, gene of interest, and 3'UTR being operably linked from 5' to 3'; and

e) an intron sequence that is incorporated in said untranslated leader sequence or in said gene of interest, said intron sequence being from an intron of a maize gene that is preferentially expressed in said selected tissue.

A related embodiment of the invention is a recombinant gene cassette competent for effecting constitutive expression of a gene of interest in transformed maize comprising:

a) a promoter from a first maize gene, said first maize gene being one that is naturally expressed preferentially in a specific tissue;

b) an untranslated leader sequence;

c) the gene of interest, said gene being one other than said first maize gene;

d) a 3'UTR;

said promoter, untranslated sequence, gene of interest, and 3'UTR being operably linked from 5' to 3'; and

e) an intron sequence that is incorporated in said untranslated leader or in said gene of interest, said intron sequence being from an intron of a maize gene that is naturally expressed constitutively.

In a particular embodiment the intron is one from the maize *Adh1* expressed gene, and the resulting recombinant gene cassette provides constitutive expression in maize.

In another of its aspects, the invention provides DNA constructs comprising, operatively linked in the 5' to 3' direction,

a) a promoter having as at least part of its sequence bp 4086-4148 bp of SEQ ID NO 1;

b) an untranslated leader sequence comprising bp 4149-4200 of SEQ ID NO 1,

c) a gene of interest not naturally associated with said promoter, and

d) a 3'UTR.

Preferred embodiments of this aspect of the invention are those wherein the promoter comprises bp 3187 to 4148, bp 2532-4148, or bp 1-4148 of SEQ ID NO 1. Particularly preferred are each of the preferred embodiments wherein said 3'UTR has the sequence of bp 6066-6340 or bp 6066-6439 of SEQ ID NO 1.

In another of its aspects, the invention provides DNA constructs comprising, operatively linked in the 5' to 3' direction,

a) a promoter having as at least part of its sequence bp 4086-4148 bp of SEQ ID NO 1;

b) an untranslated leader sequence not naturally associated with said promoter,

c) a gene of interest,

d) a 3'UTR.

Preferred embodiments of this aspect of the invention are those wherein the promoter comprises bp 3187 to 4148, bp 2532-4148, or bp 1-4148 of SEQ ID NO 1. Particularly preferred are each of the preferred embodiments wherein said 3'UTR has the sequence of bp 6066-6340 or bp 6066-6439 of SEQ ID NO 1.

In another of its aspects, the invention provides a DNA construct comprising, operatively linked in the 5' to 3' direction,

a) a promoter having as at least a part of its sequence bp 4086-4148 bp of SEQ ID NO 1;

b) an untranslated leader sequence comprising bp 4149-4200 of SEQ ID NO 1;

c) an intron selected from the group consisting of an *Adh1* gene intron and bp 4426-5058 of SEQ ID NO 1;

d) a gene of interest; and

e) a 3'UTR.

Preferred embodiments of this aspect of the invention are again those wherein the promoter comprises bp 3187 to 4148, bp 2532-4148, or bp 1-4148 of SEQ ID NO 1. Particularly preferred are each of the preferred embodiments wherein said 3'UTR has the sequence of bp 6066-6340 or bp 6066-6439 of SEQ ID NO 1.

In another of its aspects, the invention provides a DNA construct comprising, in the 5' to 3' direction,

a) a promoter having as at least part of its sequence bp 4086-4148 bp of SEQ ID NO 1;

b) an untranslated leader sequence;

c) an intron selected from the group consisting of an *Adh1* gene intron and bp 4426-5058 of SEQ ID NO 1;

d) a cloning site;

e) a 3'UTR.

In accordance with another significant aspect of the invention, there is provided a recombinant gene cassette comprised of the following operably linked sequences, from 5' to

3': a promoter; an untranslated leader sequence; a gene of interest; and the *per5* 3'UTR, bp 6068-6431 of SEQ ID NO 1.

In another of its aspects, the invention provides a plasmid comprising a promoter having as at least part of its sequence bp 4086-4148 of SEQ ID NO 1.

In another of its aspects, the invention provides a transformed plant comprising at least one plant cell that contains a DNA construct of the invention. The plant may be a monocot or dicot. Preferred plants are maize, rice, cotton and tobacco.

In another of its aspects, the invention provides seed or grain that contains a DNA construct of the invention.

Detailed Description of the Invention

In one of its aspects, the present invention relates to regulatory sequences derived from the maize root preferential cationic peroxidase protein (*per5*) that are able to regulate expression of associated DNA sequences in plants. More specifically, the invention provides novel promoter sequences and constructs using them. It also provides novel DNA constructs utilizing the *per5* untranslated leader and/or 3'UTR. It also provides novel DNA constructs utilizing the introns from the *per5* gene.

The DNA sequence for a 6550 bp fragment of the genomic clone of the maize root-preferential cationic peroxidase gene is given in SEQ ID NO 1. The sequence includes a 5' flanking region (nt 1-4200), of which nucleotides 4149-4200 correspond to the untranslated leader sequence. The coding sequence for the maize root-preferential cationic peroxidase is composed of four exons: exon 1 (nt 4201-4425), exon 2 (nt 5059-5250), exon 3 (nt 5383-5547), and exon 4 (nt 5649-6065). It should be noted that the first 96 nucleotides of exon 1 (nt 4201-4296) code for a 32 amino acid signal peptide, which is excised from the polypeptide after translation to provide the mature protein. Three introns were found: intron 1 (nt 4426-5058), intron 2 (5251-5382), and intron 3 (5548-5648). The 3' flanking region (373 nucleotides in length) extends from nucleotide 6069 (after the UGA codon at nucleotides 6066-6068) to nucleotide 6550, including a polyadenylation signal at nucleotides 6307-6312.

We have discovered that promoters derived from certain tissue preferential maize genes require the presence of an intron in the transcribed portion of the gene in order for them to provide effective expression in maize and that the temporal and tissue specificity observed depends on the intron used. A recombinant gene cassette having a tissue

preferential maize promoter, but lacking an intron in the transcribed portion of the gene, does not give appropriate expression in transformed maize. If the transcribed portion of the cassette includes an intron derived from a maize gene of similar tissue specificity to the maize gene from which the promoter was obtained, the gene cassette will restore tissue preferential expression in maize. The intron may be, but need not necessarily be, from the same gene as the promoter. If an intron derived from another maize gene, such as *Adh1* intron 1, is used in a gene cassette with a promoter from a tissue preferential maize gene, the cassette will give generally constitutive expression in maize. We have also found that these considerations apply to transgenic maize, but not to transgenic rice. Tissue preferential maize promoters can be used to drive recombinant genes in rice without an intron.

In accordance with the foregoing unexpected and significant findings, the present invention provides a recombinant gene cassette competent for effecting preferential expression of a gene of interest in a selected tissue of transformed maize, said gene cassette comprising:

a) a promoter from a first maize gene, said first maize gene being one that is naturally expressed preferentially in the selected tissue;

b) an untranslated leader sequence;

c) the gene of interest, said gene being one other than said first maize gene;

d) a 3'UTR;

said promoter, untranslated sequence, gene of interest, and 3'UTR being operably linked from 5' to 3'; and

e) an intron sequence that is incorporated in said untranslated leader sequence or in said gene of interest, said intron sequence being from an intron of a maize gene that is preferentially expressed in said selected tissue.

The promoter used in this embodiment can be from any maize gene that is preferentially expressed in the tissue of interest. Such maize genes can be identified by conventional methods, for example, by techniques involving differential screening of mRNA sequences.

A detailed example of identification and isolation of a tissue preferential maize gene is given herein for the root preferential maize cationic peroxidase gene. The method illustrated in this example can be used to isolate additional genes from various maize tissues.

Examples of tissue preferential maize genes that have promoters suitable for use in the invention include: O-methyl transferase and glutamine synthetase 1.

A preferred promoter is the *per5* promoter, i.e. the promoter from the root preferential maize cationic peroxidase gene. Particularly preferred is the promoter comprising bp 1 to 4215 of SEQ ID NO 1.

The non-translated leader sequence can be derived from any suitable source and may be specifically modified to increase the translation of the mRNA. The 5' non-translated region may be obtained from the promoter selected to express the gene, the native leader sequence of the gene or coding region to be expressed, viral RNAs, suitable eukaryotic genes, or may be a synthetic sequence.

The gene of interest may be any gene that it is desired to express in plants. Particularly useful genes are those that confer tolerance to herbicides, insects, or viruses, and genes that provide improved nutritional value or processing characteristics of the plant. Examples of suitable agronomically useful genes include the insecticidal gene from *Bacillus thuringiensis* for conferring insect resistance and the 5'-enolpyruvyl-3'-phosphoshikimate synthase (EPSPS) gene and any variant thereof for conferring tolerance to glyphosate herbicides. Other suitable genes are identified hereinafter. As is readily understood by those skilled in the art, any agronomically important gene conferring a desired trait can be used.

The 3' UTR, or 3' untranslated region, that is employed is one that confers efficient processing of the mRNA, maintains stability of the message and directs the addition of adenosine ribonucleotides to the 3' end of the transcribed mRNA sequence. The 3' UTR may be native with the promoter region, native with the structural gene, or may be derived from another source. Suitable 3' UTRs include but are, not limited to: the *per5* 3' UTR, and the 3' UTR of the nopaline synthase (*nos*) gene.

The intron used will depend on the particular tissue in which it is desired to preferentially express the gene of interest. For tissue preferential expression in maize, the intron should be selected from a maize gene that is naturally expressed preferentially in the selected tissue.

The intron must be incorporated into a transcribed region of the cassette. It is preferably incorporated into the untranslated leader 5' of the gene of interest and 3' of the promoter or within the translated region of the gene.

Why certain tissue preferential maize genes require an intron to enable effective expression in maize tissues is not known, but experiments indicate that the critical event is post-transcriptional processing. Accordingly, the present invention requires that the intron be provided in a transcribed portion of the gene cassette.

A related embodiment of the invention is a recombinant gene cassette competent for effecting constitutive expression of a gene of interest in transformed maize comprising:

a) a promoter from a first maize gene, said first maize gene being one that is naturally expressed preferentially in a specific tissue;

b) an untranslated leader sequence;

c) the gene of interest, said gene being one other than said first maize gene;

d) a 3'UTR;

said promoter, untranslated sequence, gene of interest, and 3'UTR being operably linked from 5' to 3'; and

e) an intron sequence that is incorporated in said untranslated leader or in said gene of interest, said intron sequence being from an intron of a maize gene that is naturally expressed constitutively.

This embodiment differs from the previous embodiment in that the intron is one from a gene expressed in most tissues, and the expression obtained from the resulting recombinant gene cassette in maize is constitutive. Suitable introns for use in this embodiment of the invention include *Adh1* intron 1, Ubiquitin intron 1, and Bronze 2 intron 1. Particularly preferred is the *Adh1* intron 1. Although it has previously been reported that the *Adh1* intron 1 is able to enhance expression of constitutively expressed genes, it has never been reported or suggested that the *Adh1* intron can alter the tissue preferential characteristics of a tissue preferential maize promoter.

The present invention is generally applicable to the expression of structural genes in both monocotyledonous and dicotyledonous plants. This invention is particularly suitable for any member of the monocotyledonous (monocot) plant family including, but not limited to, maize, rice, barley, oats, wheat, sorghum, rye, sugarcane, pineapple, yams, onion, banana, coconut, and dates. A preferred application of the invention is in production of transgenic maize plants.

This invention, utilizing a promoter constructed for monocots, is particularly applicable to the family *Graminaceae*, in particular to maize, wheat, rice, oat, barley and sorghum.

In accordance with another aspect of the invention, there is provided a recombinant gene cassette comprised of: a promoter; an untranslated leader sequence; a gene of interest; and the *per5* 3'UTR. Use of the *per5* 3'UTR provides enhanced expression compared to similar gene cassettes utilizing the *nos* 3'UTR.

The promoter used with the *per5* 3'UTR can be any promoter suitable for use in plants. Suitable promoters can be obtained from a variety of sources, such as plants or plant DNA viruses. Preferred promoters are the *per5* promoter, the 35T promoter (described hereinafter in Examples 20 and 23), and the ubiquitin promoter. Useful promoters include those isolated from the caulimovirus group, such as the cauliflower mosaic virus 19S and 35S (CaMV19S and CaMV35S) transcript promoters. Other useful promoters include the enhanced CaMV35S promoter (eCaMV35S) as described by Kat *et al.* (1987) and the small subunit promoter of ribulose 1,5-bisphosphate carboxylase oxygenase (RUBISCO). Examples of other suitable promoters are rice actin gene promoter; cyclophilin promoter; *Adh1* gene promoter, Callis *et al.* (1987); Class I patatin promoter, Bevan *et al.* (1986); ADP glucose pyrophosphorylase promoter; .beta.-conglycinin promoter, Tierney *et al.* (1987); E8 promoter, Deikman *et al.* (1988); 2AII promoter, Pear *et al.* (1989); acid chitinase promoter, Samac *et al.* (1990). The promoter selected should be capable of causing sufficient expression of the desired protein alone, but especially when used with the *per5* 3'UTR, to result in the production of an effective amount of the desired protein to cause the plant cells and plants regenerated therefrom to exhibit the properties which are phenotypically caused by the expressed protein.

The untranslated leader used with the *per5* 3'UTR is not critical. The untranslated leader will typically be one that is naturally associated with the promoter. The untranslated leader may be one that has been modified in accordance with another aspect of the present invention to include an intron. It may also be a heterologous sequence, such as one provided by US Patent No. 5,362,865. This non-translated leader sequence can be derived from any suitable source and can be specifically modified to increase translation of the mRNA.

The gene of interest may be any gene that it is desired to express in plants, as described above.

The terms "per5 3'UTR" and/or "per5 transcription termination region" are intended to refer to a sequence comprising bp 6068 to 6431of SEQ ID NO 1.

Construction of gene cassettes utilizing the *per5* 3'UTR is readily accomplished utilizing well known methods, such as those disclosed in Sambrook *et al.* (1989); and Ausubel *et al.* (1987).

As used in the present application, the terms "root-preferential promoter", "root-preferential expression", "tissue-preferential expression" and "preferential expression" are used to indicate that a given DNA sequence derived from the 5' flanking or upstream region of a plant gene of which the structural gene is expressed in the root tissue exclusively, or almost exclusively and not in the majority of other plant parts. This DNA sequence when connected to an open reading frame of a gene for a protein of known or unknown function causes some differential effect; i.e., that the transcription of the associated DNA sequences or the expression of a gene product is greater in some tissue, for example, the roots of a plant, than in some or all other tissues of the plant, for example, the seed. Expression of the product of the associated gene is indicated by any conventional RNA, cDNA, protein assay or biological assay, or that a given DNA sequence will demonstrate.

This invention involves the construction of a recombinant DNA construct combining DNA sequences from the promoter of a maize root-preferential cationic peroxidase gene, a plant expressible structural gene (e.g. the GUS gene (Jefferson, (1987)) and a suitable terminator.

The present invention also includes DNA sequences having substantial sequence homology with the specifically disclosed regulatory sequences, such that they are able to have the disclosed effect on expression.

As used in the present application, the term "substantial sequence homology" is used to indicate that a nucleotide sequence (in the case of DNA or RNA) or an amino acid sequence (in the case of a protein or polypeptide) exhibits substantial, functional or structural equivalence with another nucleotide or amino acid sequence. Any functional or structural differences between sequences having substantial sequence homology will be *de minimis*; that is they will not affect the ability of the sequence to function as indicated in the present application. For example, a sequence which has substantial sequence homology with a DNA sequence disclosed to be a root-preferential promoter will be able to direct the root-preferential expression of an associated DNA sequence. Sequences that have substantial

sequence homology with the sequences disclosed herein are usually variants of the disclosed sequence, such as mutations, but may also be synthetic sequences.

In most cases, sequences having 95% homology to the sequences specifically disclosed herein will function as equivalents, and in many cases considerably less homology, for example 75% or 80%, will be acceptable. Locating the parts of these sequences that are not critical may be time consuming, but is routine and well within the skill in the art.

DNA encoding the maize root-preferential cationic peroxidase promoter may be prepared from chromosomal DNA or DNA of synthetic origin by using well-known techniques. Specifically comprehended as part of this invention are genomic DNA sequences. Genomic DNA may be isolated by standard techniques. Sambrook *et al.* (1989); Mullis *et al.* (1987); Horton *et al.* (1989); Erlich (ed.)(1989). It is also possible to prepare synthetic sequences by oligonucleotide synthesis. See Caruthers (1983) and Beaucage *et al.* (1981).

It is contemplated that sequences corresponding to the above noted sequences may contain one or more modifications in the sequences from the wild-type but will still render the respective elements comparable with respect to the teachings of this invention. For example, as noted above, fragments may be used. One may incorporate modifications into the isolated sequences including the addition, deletion, or nonconservative substitution of a limited number of various nucleotides or the conservative substitution of many nucleotides. Further, the construction of such DNA molecules can employ sources which have been shown to confer enhancement of expression of heterologous genes placed under their regulatory control. Exemplary techniques for modifying oligonucleotide sequences include using polynucleotide-mediated, site-directed mutagenesis. See Zoller *et al.* (1984); Higuchi *et al.* (1988); Ho *et al.* (1989); Horton *et al.* (1989); and PCR Technology: Principles and Applications for DNA Amplification, (ed.) Erlich (1989).

In one embodiment, an expression cassette of this invention, will comprise, in the 5' to 3' direction, the maize root-preferential cationic peroxidase promoter sequence, in reading frame, one or more nucleic acid sequences of interest followed by a transcript termination sequence. The expression cassette may be used in a variety of ways, including for example, insertion into a plant cell for the expression of the nucleic acid sequence of interest.

The tissue-preferential promoter DNA sequences are preferably linked operably to a coding DNA sequence, for example, a DNA sequence which is transcribed into RNA, or which is ultimately expressed in the production of a protein product.

A promoter DNA sequence is said to be "operably linked" to a coding DNA sequence if the two are situated such that the promoter DNA sequence influences the transcription of the coding DNA sequence. For example, if the coding DNA sequence codes for the production of a protein, the promoter DNA sequence would be operably linked to the coding DNA sequence if the promoter DNA sequence affects the expression of the protein product from the coding DNA sequence. For example, in a DNA sequence comprising a promoter DNA sequence physically attached to a coding DNA sequence in the same chimeric construct, the two sequences are likely to be operably linked.

The DNA sequence associated with the regulatory or promoter DNA sequence may be heterologous or homologous, that is, the inserted genes may be from a plant of a different species than the recipient plant. In either case, the DNA sequences, vectors and plants of the present invention are useful for directing transcription of the associated DNA sequence so that the mRNA transcribed or the protein encoded by the associated DNA sequence is expressed in greater abundance in some plant tissue, such as the root, leaves or stem, than in the seed. Thus, the associated DNA sequence preferably may code for a protein that is desired to be expressed in a plant only in preferred tissue, such as the roots, leaves or stems, and not in the seed.

Promoters are positioned 5' (upstream) to the genes that they control. As is known in the art, some variation in this distance can be accommodated without loss of promoter function. Similarly, the preferred positioning of a regulatory sequence element with respect to a heterologous gene to be placed under its control is defined by the positioning of the element in its natural setting, i.e., the genes from which it is derived. Again, as is known in the art and demonstrated herein with multiple copies of regulatory elements, some variation in this distance can occur.

Any plant-expressible structural gene can be used in these constructions. A structural gene is that portion of a gene comprising a DNA segment encoding a protein, polypeptide, antisense RNA or ribozyme or a portion thereof. The term can refer to copies of a structural gene naturally found within the cell, but artificially introduced, or the structural gene may

encode a protein not normally found in the plant cell into which the gene is introduced, in which case it is termed a heterologous gene.

The associated DNA sequence may code, for example, for proteins known to inhibit insects or plant pathogens such as fungi, bacteria and nematodes. These proteins include, but are not limited to, plant non-specific lipid acyl hydrolases, especially patatin; midgut-effective plant cystatins, especially potato papain inhibitor; magainins, Zasloff (1987); cecropins, Hultmark *et al.* (1982); attacins, Hultmark *et al.* (1983); melittin; gramicidin S, Katsu *et al.* (1988); sodium channel proteins and synthetic fragments, Oiki *et al.* (1988): the alpha toxin of *Staphylococcus aureus*, Tobkes *et al.* (1985); apolipoproteins and fragments thereof, Knott *et al.* (1985)and Nakagawa *et al.* (1985); alamethicin and a variety of synthetic amphipathic peptides, Kaiser *et al.* (1987); lectins, Lis *et al.* (1986) and Van Parijs *et al.* (1991); pathogenesis-related proteins, Linthorst (1991); osmotins and permatins, Vigers *et al.* (1992) and Woloscuk *et al.* (1991); chitinases; glucanases, Lewah *et al.* (1991); thionins, Bohlmann and Apel (1991); protease inhibitors, Ryan (1990); plant anti-microbial peptides, Cammue *et al.* (1992); and polypeptides from *Bacillus thuringiensis*, which are postulated to generate small pores in the insect gut cell membrane, Knowles *et al.* (1987) and Hofte and Whitely (1989).

The structural gene sequence will generally be one which originates from a plant of a species different from that of the target organism. However, the present invention also contemplates the root preferential expression of structural genes which originates from a plant of the same species as that of the target plant but which are not natively expressed under control of the native root preferential cationic peroxidase (*per5*) promoter.

The structural gene may be derived in whole or in part from a bacterial genome or episome, eukaryotic genomic, mitochondrial or plastid DNA, cDNA, viral DNA, or chemically synthesized DNA. It is possible that a structural gene may contain one or more modifications in either the coding or the untranslated regions which could affect the biological activity or the chemical structure of the expression product, the rate of expression, or the manner of expression control. Such modifications include, but are not limited to, mutations, insertions, deletions, rearrangements and substitutions of one or more nucleotides. The structural gene may constitute an uninterrupted coding sequence or it may include one or more introns, bounded by the appropriate plant-functional splice junctions. The structural gene may be a composite of segments derived from a plurality of sources, naturally

occurring or synthetic. The structural gene may also encode a fusion protein, so long as the experimental manipulations maintain functionality in the joining of the coding sequences.

The use of a signal sequence to secrete or sequester in a selected organelle allows the protein to be in a metabolically inert location until released in the gut environment of an insect pathogen. Moreover, some proteins are accumulated to higher levels in transgenic plants when they are secreted from the cells, rather than stored in the cytosol. Hiatt, *et al.* (1989).

At the 3' terminus of the structural gene will be provided a termination sequence which is functional in plants. A wide variety of termination regions are available that may be obtained from genes capable of expression in plant hosts, e.g., bacterial, opine, viral, and plant genes. Suitable 3' UTRs include those that are known to those skilled in the art, such as the *nos* 3', *tmL* 3', or *acp* 3', for example.

In preparing the constructs of this invention, the various DNA fragments may be manipulated, so as to provide for the DNA sequences in the proper orientation and, as appropriate, in the proper reading frame. Adapters or linkers may be employed for joining the DNA fragments or other manipulations may be involved to provide for convenient restriction sites, removal of superfluous DNA, removal of restriction sites, or the like.

In carrying out the various steps, cloning is employed, so as to amplify a vector containing the promoter/gene of interest for subsequent introduction into the desired host cells. A wide variety of cloning vectors are available, where the cloning vector includes a replication system functional in *E. coli* and a marker which allows for selection of the transformed cells. Illustrative vectors include pBR322, pUC series, pACYC184, Bluescript series (Stratagene) etc. Thus, the sequence may be inserted into the vector at an appropriate restriction site(s), the resulting plasmid used to transform the *E. coli* host (e.g., *E. coli* strains HB101, JM101 and DH5α), the *E. coli* grown in an appropriate nutrient medium and the cells harvested and lysed and the plasmid recovered. Analysis may involve sequence analysis, restriction analysis, electrophoresis, or the like. After each manipulation the DNA sequence to be used in the final construct may be restricted and joined to the next sequence, where each of the partial constructs may be cloned in the same or different plasmids.

Vectors are available or can be readily prepared for transformation of plant cells. In general, plasmid or viral vectors should contain all the DNA control sequences necessary for both maintenance and expression of a heterologous DNA sequence in a given host. Such

control sequences generally include, in addition to the maize root-preferential cationic peroxidase promoter sequence (including a transcriptional start site), a leader sequence and a DNA sequence coding for translation start-signal codon (generally obtained from either the maize root-preferential cationic peroxidase gene or from the gene of interest to be expressed by the promoter or from a leader from a third gene which is known to work well or enhance expression in the selected host cell), a translation terminator codon, and a DNA sequence coding for a 3' non-translated region containing signals controlling messenger RNA processing. Selection of appropriate elements to optimize expression in any particular species is a matter of ordinary skill in the art utilizing the teachings of this disclosure; in some cases hybrid constructions are preferred, combining promoter elements upstream of the tissue preferential promoter TATA and CAAT box to a minimal 35S derived promoter consisting of the 35S TATA and CAAT box. Finally, the vectors should desirably have a marker gene that is capable of providing a phenotypical property which allows for identification of host cells containing the vector, and an intron in the 5' untranslated region, e.g., intron 1 from the maize alcohol dehydrogenase gene that enhances the steady state levels of mRNA of the marker gene.

The activity of the foreign gene inserted into plant cells is dependent upon the influence of endogenous plant DNA adjacent the insert. Generally, the insertion of heterologous genes appears to be random using any transformation technique; however, technology currently exists for producing plants with site specific recombination of DNA into plant cells (see WO/9109957). The particular methods used to transform such plant cells are not critical to this invention, nor are subsequent steps, such as regeneration of such plant cells, as necessary. Any method or combination of methods resulting in the expression of the desired sequence or sequences under the control of the promoter is acceptable.

Conventional technologies for introducing biological material into host cells include electroporation, as disclosed in Shigekawa and Dower (1988), Miller, *et al.* (1988), and Powell, *et al* (1988); direct DNA uptake mechanisms, as disclosed in Mandel and Higa (1972) and Dityatkin, *et al.* (1972), Wigler, *et al.* (1979) and Uchimiya, *et al.* (1982); fusion mechanisms, as disclosed in Uchidaz, *et al.* (1980); infectious agents, as disclosed in Fraley, *et al.* (1986) and Anderson (1984); microinjection mechanisms, as disclosed in Crossway, *et al.* (1986); and high velocity projectile mechanisms, as disclosed in EPO 0 405 696.

Plant cells from monocotyledonous or dicotyledonous plants can be transformed according to the present invention. Monocotyledonous species include barley, wheat, maize, oat and sorghum and rice. Dicotyledonous species include tobacco, tomato, sunflower, cotton, sugarbeet, potato, lettuce, melon, soybean and canola (rapeseed).

The appropriate procedure to transform a selected host cell may be chosen in accordance with the host cell used. Based on the experience to date, there appears to be little difference in the expression of genes, once inserted into cells, attributable to the method of transformation itself. Once introduced into the plant tissue, the expression of the structural gene may be assayed in a transient expression system, or it may be determined after selection for stable integration within the plant genome.

Techniques are known for the *in vitro* culture of plant tissue, and in a number of cases, for regeneration into whole plants. The appropriate procedure to produce mature transgenic plants may be chosen in accordance with the plant species used. Regeneration varies from species to species of plants. Efficient regeneration will depend upon the medium, on the genotype and on the history of the culture. Once whole plants have been obtained, they can be sexually or clonally reproduced in such a manner that at least one copy of the sequence is present in the cells of the progeny of the reproduction. Seed from the regenerated plants can be collected for future use, and plants grown from this seed. Procedures for transferring the introduced gene from the originally transformed plant into commercially useful cultivars are known to those skilled in the art.

Example 1
Characterization Of A Maize Root-Preferential Cationic Peroxidase

The presence of peroxidase activity can be detected *in situ* in sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) by incubation with H_2O_2 and a chromogenic substrate such as 3,3'-diaminobenzidine. Tissue specific peroxidase activity was detected by extraction of proteins from root, stem and leaf tissue of maize followed by detection in gels according to Nakamura *et al.* (see Nakamura *et al.* (1988)) essentially as follows. One gram of maize tissue was macerated in mortar in 1 mL extraction buffer, composed of 62.5 mM TrisHCl pH 6.8, 5 mM $MgCl_2$, 0.5 M sucrose, and 0.1% ascorbic acid, centrifuged and passed over 0.2 µM filter to remove plant debris. Total protein was determined using the Bradford protein assay. See Bradford (1976). Ten micrograms of protein of each tissue was

electrophoresed on a SDS-poly acrylamide gel. Beta-mercaptoethanol was omitted from the sample buffer to retain enzyme activity. Following electrophoresis the gel was washed two times in 50 mM TrisHCl pH 7.5 for 30 minutes each to remove SDS, and then incubated in the assay solution, which was composed of 50 mM TrisHCl pH 7.5, 0.5 mg/mL diamino benzidine and 0.01% hydrogen peroxide for 10 minutes. Bands corresponding to peroxidase activity were visualized by the formation of a brown precipitate. Non-reduced molecular weight markers (Amersham Corporation) were run in a parallel lane and visualized by standard protein staining in a separate incubation with Coomassie Brilliant Blue. Peroxidase activity in the gel corresponding to a band migrating at approximately 44 kD was only detected in root tissue and was not present in either leaf or stem tissue. Identical patterns of peroxidase staining were produced when several different maize genotypes were examined for root-specific peroxidase isozymes (B37 x H84, Pioneer Hybrid 3737, B73).

Example 2

Isolation Of cDNA Clones Encoding The Maize Root-Preferential Cationic Peroxidase

A. RNA isolation, cDNA synthesis and library construction.

Maize kernels (Zea mays hybrid B37 x H84) were germinated on filter paper under sterile conditions. At 6 days post germination root tissue was harvested and frozen in liquid nitrogen and ground in a mortar and pestle until a fine powder was obtained. The powder was added to 10 mLs of TLE buffer (0.2 M Tris HCl pH 8.2, 0.1 M LiCl, 5 mM EDTA) containing 1% SDS and extracted with 50 mLs of TLE equilibrated phenol and 50 mLs of chloroform. The extraction was incubated on ice for 45 minutes with shaking, and subsequently incubated at 50°C for 20 minutes. The aqueous phase was transferred to a clean centrifuge tube following centrifugation, and reextracted twice with one half volume of phenol/chloroform (1:1), followed by extractions with chloroform. RNA was precipitated from the aqueous phase by addition of one third volume of 8 M LiCl and incubation at 4°C for 24 hrs. The precipitate was collected by centrifugation, washed with 2M LiCl and resuspended in 12 mLs of water. RNA was reprecipitated by addition of an equal volume of 4 M LiCl, incubation at 4°C for 24 hrs and centrifugation. The RNA pellet was resuspended in 2 mL of water and ethanol precipitated by addition of 200 µl 3 M Na Acetate and 5.5 mL of ethanol and 16 hr incubation at -20°C, followed by centrifugation. The final RNA pellet

was resuspended in 1 mL water. The concentration of the RNA was determined using measurement of the absorption at 260 nm. Messenger RNA was purified by binding to and subsequent elution of polyA Quickkit™ columns exactly as described by the supplier (Stratagene Cloning Systems, La Jolla, CA). The concentration was determined by A260 measurement. cDNA was synthesized from 5 micrograms of polyA+ RNA using the ZAP-cDNA® synthesis kit, cloned into the Uni-ZAP® vector, packaged into phage heads using Stratagene Gigapack Gold® packaging extracts and infected and amplified on *E. coli* strain PLK-F' exactly according to the protocols provided by the supplier (Stratagene). The titer of the resulting amplified library was determined by plating on PLK-F' cells and was determined at 2.7×10^9 plaque forming units (pfu)/mL.

B. Isolation of a peroxidase hybridization probe. A hybridization probe corresponding to a central portion of peroxidase cDNA sequences was isolated as follows. Sequence analysis of a number of cloned peroxidases indicated that there are several domains in the predicted and/or determined amino acid sequences that are highly conserved. See Lagrimini and Rothstein (1987). Two degenerate oligonucleotide primers were synthesized against two conserved domains, taking in account a bias for C or G over A or T in the third codon position in maize. Part of the first conserved domain, FHDCFVNGC corresponding to amino acids 41 through 49 of the tobacco peroxidase (see Lagrimini and Rothstein (1987)) was reverse translated into the degenerate oligonucleotide MM1: 5'-TTYCAYGAYTGYTTYGTYAAYGGBTG-3' (SEQ ID NO 3). Part of a second conserved domain, VALSGAHT (corresponding to amino acids 161 through 168 of the tobacco peroxidase (see Lagrimini and Rothstein (1987)) was reverse translated and reverse complemented to give the degenerate oligonucleotide MM3: 5'-SGTRTGSGCSCCGSWSAGVGCSAC-3' (SEQ ID NO 4). In both oligonucleotides, Y indicates the degeneracy C and T; R indicates A and G, S indicates C and G; W indicates A and T; V indicates A, C, and G; and B indicates C ,G, and T;

Using the Polymerase Chain Reaction™ kit (Perkin Elmer Cetus) a 380 bp DNA fragment was amplified using total root cDNA library DNA as template. The size of this fragment corresponded well to the expected size based on the distance of the two domains in peroxidase proteins, 128 amino acids corresponding to 384 nt. Following gel purification the 380 nt fragment was radiolabeled using random primer labeling with an Oligo

Labeling™ kit (Pharmacia LKB Biotechnology, Inc, Piscataway, NJ) as per the supplier's instructions with [D1]50 microCuries [α-^{32}P]dCTP.

C. Screening of the root cDNA library. Two hundred thousand phages were plated on *E. coli* XL1 Blue cells (Stratagene) divided over ten plates. Duplicate plaque lift filters were made of each plate. Filters were prehybridized and hybridized in a total volume of 150 mLs of hybridization solution according to standard procedures (Sambrook *et al.* 1989). The approximate concentration of labelled probe in the hybridization was 2.20 x 10^5 cpm/mL. Following hybridization filters were washed according to standard procedures, air dried, covered and exposed to Kodak XAR5 film. Signals were determined positive if they occurred in the same position on the two duplicate filters of one plate relative to the markings. Putative positive phage were cored out of the plate and stored in 1 mL of SM buffer. Thirty four positive phage were rescreened twice to obtain a pure phage stock using similar hybridization experiments as described above. DNA from all 34 positive phage cDNA clones was prepared by alkaline lysis minipreps following *in vivo* rescue of phagemids according to the protocol provided by the supplier (Stratagene) and digested with *Eco*RI and *Xho*I to release inserts. All plasmids contained one insert in the size range of 1.3-1.4 kb which hybridized with the 380 nt peroxidase probe.

Example 3

Analysis of maize root-preferential cationic peroxidase cDNA clone per5.

A. Analysis of expression pattern by Northern hybridization. RNA was prepared from root, stem, leaf, kernel and tassel tissue as described in Example 2, section A. Thirty micrograms of denatured total RNA of each tissue was electrophoresed on a 1% agarose/Na phosphate gel and transferred to nylon membrane and prehybridized and hybridized with the labeled 380 nt peroxidase probe according to standard procedures. A ~1470 nt transcript was detected in root and stem RNA, but was absent from leaf, kernel and tassel RNA. The level of the detected transcript in roots was at least 5.5 fold higher than in stem tissue.

B. Sequence analysis of the *per5* cDNA clone. Both strands of dsDNA from the cDNA clone with the longest insert (*per5*) were sequenced using the Sequenase™ sequencing kit (United States Biochemical, Cleveland, OH). Sequencing was started using the T3 and T7 primers and completed by walking along the DNA using sequencing primers

designed based on sequence derived in previous runs. The sequence of the *per5* cDNA insert is shown in SEQ ID NO 5. The *per5* cDNA insert is 1354 nucleotides (nt) in length and has a 5'-untranslated leader of 52 nt and a 275 nt 3' untranslated sequence before the start of polyadenylation. It also contains the animal consensus polyadenylation signal sequence AATAAA 34 nucleotides prior to the addition of a 28 nucleotide poly(A) tail. The cDNA has an open reading frame of 999 bp, which spans between nucleotides 53 and 1051. The first ATG codon in the cDNA sequence was chosen as the start of translation. The predicted size of the mature maize peroxidase is 301 amino acids with a MW of 32,432 and an estimated pI of 9.09. The N-terminus of the mature protein was assigned by alignment of the maize amino acid sequence with other published sequences and known N-terminal sequences obtained by N-termal amino acid sequencing. It is predicted from the cDNA sequence that the protein is initially synthesized as a preprotein of MW 35,685 with a 32-amino acid signal sequence that is 72% hydrophobic. The presence of this signal sequence, which has also been observed in several other plant peroxidases, suggests that the protein is taken up in the endoplasmic reticulum and modified for sub-cellular targeting or secretion. This is supported by the presence of four potential N-glycosylation sites (Asn-Xaa-Thr/Ser), which are at residues 53, 138, 181 and 279 of the putative mature protein. The presence of four putative N-glycosylation sites suggest a role for post-translational modification (eg. glycosylation) and explains the discrepancy in the observed (~44 kD) and predicted size of the mature protein (~36 kD). Comparison of the deduced amino acid sequences of the maize *per5* cDNA with the published sequences of wheat (see Hertig *et al.* (1991)), horseradish [C1] (see Fujiyama *et al.* (1988)), turnip [TP7] (see Mazza and Welinder (1980)), peanut [PNC1] (see Buffard *et al.* (1990)), tobacco (see Lagrimini *et al.* (1987)), and cucumber (see Morgens *et al.* (1990)) confirms that *per5* encodes a peroxidase protein. There is >80% to >92% sequence similarity between these seven plant peroxidases in four conserved domains. All seven peroxidases have eight cysteines, conserved in position in the primary sequence. These cysteines in the horseradish and turnip enzymes have been shown to be involved in intramolecular disulfide linkages.

Example 4
Isolation of the maize root-preferential cationic peroxidase genomic clone

A. Genomic DNA Blot Hybridization. Genomic DNA was isolated from a maize diploid, homozygous line (B73). The DNA was digested with the restriction enzymes *Eco*RI, *Hind*III, and *Sac*I, fractionated on a 1% agarose gel, subjected to transfer to membrane and hybridization to both a ^{32}P-labeled *per5* full-length cDNA and a *per5* cDNA gene-specific probe (GSP5). The 136 bp GSP5 probe was amplified by PCR using the *per5* cDNA clone as template DNA and primers MM21: 5'-GTCATAGAACTGTGGG -3'(SEQ ID NO 6); and MM22: 5'-ATAACATAGTACAGCG-3' (SEQ ID NO 7). This probe is composed of nt 25 - 160 of the *per5* cDNA clone and includes 27 bp of the 5' untranslated sequence, the entire coding sequence for the putative endoplasmic reticulum signal peptide and 7 bp which code for the amino-terminus of the putative *per5* mature domain.

Using the *per5* cDNA full length probe two strong hybridization signals were detected in each digest. This suggested that the *per5* gene may be present in two copies per haploid genome. However, using GSP5 as a probe only one band per lane was detected which suggested that there is only one copy of the *per5* gene per haploid genome and that the other hybridizing band on the genomic DNA blot corresponds to more distantly related sequences. This also demonstrated that probe GSP5 was gene specific and would be suitable for the isolation of the peroxidase genomic clone from a maize genomic library.

B. Isolation of the root-preferential cationic peroxidase gene from a maize W22 library. Approximately 2 x 10^6 plaques of a maize W22 genomic library (Clontech Laboratories, Inc., Palo Alto, CA) were screened using GSP5 as the probe according to standard protocol for library screening. GSP5 was used as probe because it would recognize only the genomic clones corresponding to the *per5* cDNA clone. Ten genomic clones were isolated and plaque purified. The clones were plate amplified to increase their titers, liquid lysates were grown up and phage DNA was isolated from these cultures. Restriction analysis on nine of the ten clones using *Sal*I, which liberates the genomic DNA inserts from the phage arms, showed that eight of the nine clones had the same *Sal*I banding pattern. These eight clones contained ~14.9 Kb inserts which could be cut into two *Sal*I fragments of ~10.4 Kb and ~4.5 Kb, respectively. The ninth clone (perGEN19) contained an ~15.6 Kb insert which upon *Sal*I digestion yields two fragments, ~13.1 Kb and ~2.5 Kb in size. Restriction and DNA hybridization analysis suggest that perGEN19 contains an insert which

overlaps with the *Sau*3A inserts of the other 8 clones. A representative of the eight identical genomic clones (perGEN1) was further analyzed. The ~10.4 Kb fragment was subcloned into the *Sal*I site of the plasmid pBluescript®II SK(-) (Stratagene, Inc.) generating plasmid perGEN1(10.44). Restriction digests (using *Apa*I, *Bam*HI, *Eco*RI, *Hind*III, *Kpn*I, *Nco*I, *Sac*I, and *Xba*I) and DNA blot hybridization analyses (using either the full-length *per5* cDNA or GSP5 as probes) indicated that the 10.44 Kb *Sal*I fragment on perGEN1 contained the peroxidase sequences. Further restriction digests using single and double digests of *Hind*III, *Kpn*I, *Sac*I, and *Xba*I and DNA blot hybridization analyses using gel-purified *Kpn*I perGEN1(10.44) fragments as probes was performed on perGEN1(10.44).

Example 5
Sequence of the maize root-preferential cationic peroxidase gene

A total of 6550 nt of genomic sequence covering the maize root-preferential cationic peroxidase gene and its 5' and 3' flanking sequences was obtained by sequencing overlapping subfragments of plasmid perGEN1(10.44) which hybridized with the GSP5 probe described in Example 3 as well as the *per5* cDNA insert. The sequence is shown in SEQ ID NO 1. The sequencing procedures were standard techniques known to those skilled in the art. The upstream flanking region from the 5'-most *Nco*I site to the putative start site of translation was determined to be 4200 nt in length. The maize root-preferential cationic peroxidase gene is composed of exons: exon 1 (225 bp), exon 2 (192 bp), exon 3 (166 bp), and exon 4 (416 bp). The GC-content of the exons is 54.7%. The sequence of the compiled exon sequences was 100% identical to that of the coding region for the *per5* cDNA. Translation of these exons resulted in a deduced protein sequence that is 100% identical to the deduced protein sequence for the *per5* cDNA sequence. Three introns were found: intron 1 (633 bp, %AU = 62.7, %U = 33.8), intron 2 (132 bp, %AU = 63.6, %U = 35.6), and intron 3 (101 bp, %AU = 65.3, %U = 37.6). The downstream flanking region from the UGA codon to the 3' most *Xba*I site was found to be 373 bp in length. The intron splice sites did not fit the putative monocot 5' and 3' splice site consensus sequences perfectly, but did follow the mammalian "GU/AG rule" for splice sites. The intron sequences also conformed to the definition of maize intron sequences suggested by Walbot. See Walbot *et al.* (1991).

Example 6
pDAB 406

This Example describes pDAB 406, a vector designed for testing of promoter activity in both transient and stable transformation experiments. The complete sequence for pDAB 406 is given in SEQ ID NO 8. With reference to SEQ ID NO 8, significant features of pDAB 406 are given in Table 1.

Table 1: Features of pDAB 406

nt (SEQ ID NO 8)	Features
1-6	*Apa*I site
7-24	multiple cloning site (*Nhe*I, *Kpn*I, *Sma*I)
25-30	SalI site
32-1840	*E. coli uidA* reporter gene encoding the beta-glucuronidase protein (GUS) from pKA382 and TGA stop codon
1841-1883	3' untranslated region from pBI221
1894-1899	*Sst*I site
1900-2168	nopaline synthetase 3' polyA sequence (*nos* 3'UTR)
2174-2179	*Hin*dIII site
2180-2185	*Bgl*II site
2186-2932	a modified CaMV 35S promoter
2195-2446	MCASTRAS nt 7093-7344
2455-2801	MCASTRAS nt 7093-7439
2814-2932	Synthetic Maize Streak Virus (MSV) untranslated leader containing the maize *Adh1* intron 1
2933-2938	*Bgl*II/*Bcl*I junction
2933-3023	*Adh1*.S nt 269-359 MZEADH1.S
3024-3141	*Adh1*.S nt 704-821 MZEADH1.S
3146-3151	*Bam*HI/*Bgl*II junction
3150-3187	synthetic MSV leader containing the maize *Adh1* intron 1
3188-3193	*Nco*I
3190-4842	internal reference gene composed of the firefly luciferase gene (Lux)
4907-5165	nopaline synthetase 3' polyA sequence (*nos* 3'UTR)
5172-5177	*Bgl*II site
5178-5183	*Nde*I site
5186-5191	*Sst*I site
5195-5672	nt 6972-6495 MCASTRAS (CaMV 35S promoter)
5680-6034	nt 7089-7443 MCASTRAS (CaMV 35S promoter)
6042-7021	Tn5 nt 1539-2518; mutated 2X
6054-6848	a selectable marker gene composed of the bacterial *NPTII* gene encoding neomycin phosphotransferase which provides resistance to the antibiotics kanamycin, neomycin and G418
7022-7726	3' UTR of ORF26 gene *Agrobacterium tumifaciens* Ti plasmid (pTi 15955, nt 22438 to 21726)
7727-7732	*Nde*I site
7733-7914	pUC19 nt 1-182, reverse complement
7915-10148	nt 453 to 2686 pUC19, reverse complement
10149-10160	multiple cloning site, *Hin*dIII, *Sst*I

The vector can readily be assembled by those skilled in the art using well known methods.

Example 7

pDAB 411

This Example describes plasmid pDAB 411, which is a 11784 bp plasmid that has a pUC19 backbone and contains a gene cassette comprising 1.6kb of *per5* promoter, the *per5* untranslated leader, the GUS gene, and the *nos* 3' UTR. No intron is present in the untranslated leader of pDAB 411. The complete sequence for pDAB 411 is given in SEQ ID NO 9. With reference to SEQ ID NO 9, significant features of pDAB 411 are given in Table 2.

Table 2. Significant Features of pDAB 411

nt (SEQ ID NO 9)	Feature
1-6	*Apa*I site
7-1648	*Per5* promoter and untranslated leader sequence (corresponding to nt 2559 to 4200 of SEQ ID NO 1)
1649-1654	*Sal*I site
1656-3464	*E. coli uidA* reporter gene encoding the beta-glucuronidase protein (GUS)
3465-3507	3' untranslated region from pBI221
3518-3523	*Sst*I site
3524-3792	nopaline synthetase 3' polyA sequence (*nos* 3'UTR)
3793-11784	corresponds to 2169 to 10160 of pDAB 406 SEQ ID NO 8

Preliminary testing of pDAB 411 in transgenic maize plants failed to demonstrate appreciable GUS expression. This failure is consistent with our discovery that certain tissue preferential maize promoters require the presence of an intron in the transcribed portion of the gene for significant expression to be observed.

Example 8
pDAB 419

This Example describes construction of Plasmid pDAB 419, which is a 11991 bp plasmid that is identical to pDAB 411, except that the untranslated leader preceding the GUS gene includes a 207 bp sequence comprising a deleted version the maize *Adh1* intron 1. The complete sequence for pDAB 419 is given in SEQ ID NO 10. With reference to SEQ ID NO 10, critical features of pDAB 419 are as follows:

Table 3: Critical Features of pDAB 419

nt (SEQ ID NO 10)	Feature
1-6	*Apa*I site
7-1648	*Per5* promoter and untranslated leader sequence (corresponding to nt 2559 to 4200 of SEQ ID NO 1)
1649-1855	deleted version of maize *Adh1* intron 1 corresponding to nt 2939-3145 of SEQ ID NO 8
1856-1861	SalI site

1863-3671	*E. coli uidA* reporter gene encoding the beta-glucuronidase protein (GUS)
3672-3714	3' untranslated region from pBI221
3725-3730	*Sst*I site
3731-3999	nopaline synthetase 3' polyA sequence (*nos* 3'UTR)
4000-11991	corresponds to 2169 to 10160 of pDAB 406 SEQ-- ID NO 8

Plasmid pDAB 419 was constructed from pDAB 411 using conventional techniques. More specifically, the *per5* promoter in plasmid pDAB411 was amplified with primers MM88: 5'-ACGTACGTACGGGCCCACCACTGTTGTAACT TGTAAGCC-3' (SEQ ID NO 11) and OF192: 5' AGGCGGACCTTTGCACTGTGA GTTACCTTCGC-3'(SEQ ID NO 12). The modified *Adh1* intron 1, corresponding to nt 2939 to 3145 of SEQ ID NO 8. was amplified from plasmid pDAB406 using primers OF190: 5'-CTCTGTCGACGAGCGCAGCTGCAC GGGTC-3'(SEQ ID NO 13) and OF191: 5'-GCGAAGGTAACTCACAGTGCA AAGGTCCGCCT-3' (SEQ ID NO 14). Following amplification both fragments were purified through a 1% agarose gel. Splice Overlap Extension PCR was used to join the *per5* promoter fragment to the *Adh1* intron 1 fragment. Samples (2.5 µL) of each gel-purified fragment were mixed and re-amplified using primers MM88 and OF192 (SEQ ID NOS 11 and 12). The resulting 1.6 kB per5*adh* fragment was digested with *Apa*I and *Sal*I, gel-purified, and ligated into pDAB406 which was digested with *Apa*I and *Sal*I resulting in an 11,991 bp plasmid, pDAB419.

Example 9

Transformation of Rice with pDAB 419

This example describes transformation of rice with pDAB 419, and the histochemical and quantitative patterns of GUS expression in the transformed rice plants.

A. Transgenic Production.

1. Plant Material and Callus Culture. For initiation of embryogenic callus, mature seeds of a *Japonica* cultivar, Taipei 309 were dehusked and surface-sterilized in 70% ethanol for 2-5 min. followed by a 30-45 min soak in 50% commercial bleach (2.6% sodium hypochlorite) with a few drops of 'Liquinox' soap. The seeds were then rinsed 3 times in sterile distilled water and placed on filter paper before transferring to 'induction' media (NB). The NB medium consisted of N6 macro elements (Chu, 1978), B5 micro elements and vitamins (Gamborg *et al.*, 1968), 300 mg/L casein hydrolysate, 500 mg/L L-proline, 500 mg/L L-glutamine, 30 g/L sucrose, 2 mg/L 2,4-dichloro-phenoxyacetic acid (2,4-D), and 2.5

g/L Gelrite (Schweizerhall, NJ) with a pH adjusted to 5.8. The mature seed cultured on 'induction' media were incubated in the dark at 28° C. After 3 weeks of culture, the emerging primary callus induced from the scutellar region of mature embryo was transferred to fresh NB medium for further maintenance.

2. Plasmids and DNA Precipitation. pDAB354 containing 35T-*hpt* (hygromycin phosphotransferase providing resistance to the antibiotic hygromycin; (described in Example 25) was used in cotransformations with pDAB 419. About 140 µg of DNA was precipitated onto 60 mg of gold particles. The plasmid DNA was precipitated onto 1.5-3.0 micron (Aldrich Chemical Co., Milwaukee, WI) or 1.0 micron (Bio-Rad) gold particles. The precipitation mixture included 60 mg of pre-washed gold particles, 300 µL of water/DNA (140 µg), 74 µL of 2.5 M $CaCl_2$, and 30 µL of 0.1 M spermidine. After adding the components in the above order, the mixture was vortexed immediately, and allowed to settle for 2-3 min. Then, the supernatant was pipetted off and discarded. The DNA-coated gold particles were resuspended in 1 mL of 100% ethanol and diluted to 17.5 µg DNA/7.5 mg gold per mL of ethanol for use in blasting experiments.

3. Helium Blasting into Embryogenic Callus and Selection. Actively growing embryogenic callus cultures, 2-4 mm in size, were subjected to a high osmoticum treatment. This treatment included placing of callus on NB medium with 0.2 M mannitol and 0.2 M sorbitol (Vain *et al.*, 1993) for 4 hrs before helium blasting. Following osmoticum treatment, callus cultures were transferred to 'blasting' medium (NB+2% agar) and covered with a stainless steel screen (230 micron). Helium blasting involved accelerating the suspended DNA-coated gold particles towards and into the prepared tissue targets. The device used was an earlier prototype to the one described in US Patent #5,141,131 which is incorporated herein by reference, although both function in a similar manner. The callus cultures were blasted at different helium pressures (1,750-2,250 psi) once or twice per target. After blasting, callus was transferred back to the media with high osmoticum overnight before placing on selection medium, which consisted of NB medium with 30 mg/L hygromycin. After 2 weeks, the cultures were transferred to fresh selection medium with higher concentrations of selection agent, i.e., NB+50mg/L hygromycin (Li *et al.*, 1993).

4. Regeneration. Compact, white-yellow, embryogenic callus cultures, recovered on NB+50 mg/L hygromycin, were regenerated by transferring to 'pre-regeneration' (PR) medium + 50 mg/L hygromycin. The PR medium consisted of NB medium with 2 mg/L 6-

benzylaminopurine (BAP), 1 mg/L naphthaleneacetic acid (NAA), and 5 mg/L abscisic acid (ABA). After 2 weeks of culture in the dark, they were transferred to 'regeneration' (RN) medium. The composition of RN medium is NB medium with 3 mg/L BAP, and 0.5 mg/L NAA. The cultures on RN medium were incubated for 2 weeks at 28° C under high fluorescent light (325-ft-candles). The plantlets with 2 cm shoot were transferred to 1/2 MS medium (Murashige and Skoog, 1962) with 1/2 B5 vitamins, 10 g/L sucrose, 0.05 mg/L NAA, 50 mg/L hygromycin and 2.5 g/L Gelrite adjusted to pH 5.8 in magenta boxes. When plantlets were established with well-developed root system, they were transferred to soil (1 metromix: 1 top soil) and raised in a growth chamber or greenhouse (29/24°C day/night cycle, 50-60% humidity, 12 h photoperiod) until maturity. A total of 23 hygromycin-resistant callus lines were established.

B. GUS histochemical assays

GUS histochemical assays were conducted according to Jefferson (1987). Tissues were placed in 24-well microtitre plates (Corning, New York, NY) containing 500 µL of assay buffer per well. The assay buffer consisted of 0.1 M sodium phosphate (pH 8.0), 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 10 mM sodium EDTA, 1.9 mM 5-bromo-4-chloro-3-indolyl-beta-D-glucuronide, and 0.06% triton X-100. The plates were incubated in the dark for 1-2 days at 37° C before observations under a microscope. Fourteen of the 23 hygromycin resistant rice lines expressed the GUS gene as evidenced by blue staining after 48 hours in the GUS histochemical assay. Nine of the 14 GUS expressing lines were further characterized (Table 4).

Table 4: Histochemical GUS Staining of Transgenic Rice Callus

Line	Rating
354/419-03	++++
354/419-04	++++
354/419-07	++++
354/419-11	+++
354/419-12	++
354/419-13	+++
354/419-15	++
354/419-18	+++
354/419-21	+-

+ = Occasional blue region

+- = Light blue staining throughout

+++ = Dark blue regions

++++ = Intense blue staining throughout

C. Southern Analysis

Southern analysis was used to identify primary regenerate (Ro) plant lines from rice that contained an intact copy of the transgene and to measure the complexity of the integration event. Several leaves from each rice plant were harvested and up to five plants were sampled individually from each line. Genomic DNA from the rice Ro plants was prepared from lyophilized tissue as described by Saghai-Maroof *et al.* (1984). Eight micrograms of each DNA was digested with the restriction enzyme *Xba*I using conditions suggested by the manufacturer (Bethesda Research Laboratory, Gaithersburg, MD) and separated by agarose gel electrophoresis. The DNA was blotted onto nylon membrane as described by Southern (1975, 1980).

A probe specific for β-glucuronidase (GUS) coding region was excised from the pDAB419 plasmid using the restriction enzymes *Nco*I and *Sst*I. The resulting 1.9 kb fragment was purified with the Qiaex II DNA purification kit (Qiagen Inc., Chatsworth, CA). The probe was prepared using an oligo-labeling kit (Pharmacia LKB, Piscataway, NJ) with 50 microcuries of α^{32}P-dCTP (Amersham Life Science, Arlington Heights, IL). The GUS probe hybridized to the genomic DNA on the blots. The blots were washed at 60°C in 0.25X SSC and 0.2% SDS for 45 minutes, blotted dry and exposed to XAR-5 film overnight with two intensifying screens.

D. GUS Quantification

1. Tissue Preparation. Histochemically GUS positive plantlets, grown in Magenta boxes, were dissected into root and leaf tissues. Duplicate samples of approximately 300 mg root and 100 mg leaf were transferred to a 1.5 ml sterile sample tube (Kontes, Vineland, NJ) and placed on ice prior to freezing at -80°C. Extraction of proteins consisted of grinding tissue using a stainless steel Kontes Pellet Pestle powered by a 0.35 amp, 40 Watt motor (Model 102, Rae Corp., McHenry, IL), at a setting of "40". GUS Lysis buffer from the GUS-Light™ assay kit (Tropix, Bedford, MA) was modified with the addition of 20% glycerol to produce the extraction buffer. Before grinding, frozen samples were placed on ice and aliquots of 100 µl extraction buffer were added to the sample tube. Tissue was homogenized in approximately four 25-second intervals during which additional aliquots of extraction buffer were added for a final volume of 300 µl for root and 200 µl for leaf tissues. Samples were maintained on ice until all sample grinding was completed. Samples were then centrifuged twice at 5°C for 8 minutes at full speed (Eppendorf Centrifuge Model 5415). Supernatant was transferred to sterile microcentrifuge tubes on ice and later used to quantitate proteins and GUS; the pellet was discarded.

2. Total Protein Quantification. Quantification of extractable proteins was determined with the Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Hercules, CA). A protein standard made from bovine albumin (Sigma, St. Louis, MO) was used to obtain a standard curve from zero to 10 µg/ml. Duplicate samples for each tissue were prepared using 5 µl of protein extract with 5 µl GUS lysis buffer in a sterilized microcentrifuge tube. Water was added to bring the volume up to 800 µl before 200 µl dye reagent was added. Tubes were vortexed, then incubated at room temperature for at least 5 minutes before the liquid was transferred into 1.5 ml cuvetts and place in the spectrophotometer (Shimadzu, Japan). Absorbance measurements were made at 595 nm.

3. GUS Quantification. Analysis of GUS activity required the use of the GUS-Light™ assay kit and an automatic luminescence photometer (Model 1251 Luminometer and Model 1291 Dispenser, Bio-Orbit, Finland). For each sample, a relative level of GUS activity was measured on 1µl extract. From the initial reading, sample volumes were scaled up between 2 and 10 µl of extract per luminometer vial while remaining within the detection limits of the equipment. Samples were prepared in triplicate to which 180 µl aliquots of GUS-Light™ reaction buffer was added to each luminometer vial at 10-second intervals. After a one hour incubation at room temperature in the dark, the vials were loaded into the

sample holder of the luminometer. As each vial entered the measuring chamber, 300 µl of GUS-Light™ Light Emission Accelerator Buffer was added and luminescence was detected over a 5-second integration period. A "blank reaction" was included in the assay, using 10 µl of the GUS extraction buffer. A GUS standard, prepared to read 8,000 relative light units (RLU) from commercially available β-glucuronidase (Sigma, MO), was used to confirm the sensitivity of the equipment and reagents used. GUS readings (RLU) were corrected for the "blank" and the GUS standard readings before dividing by µg total protein.

Table 5: GUS Expression in Rice Plants Tansformed with pDAB 419

Line	Presence of Intact Construct	Number of Hybridization Products	Relative light units per mg protein	
			Root	Leaf
354/419-03	yes	10	n.d.	n.d.
354/419-04	yes	4	795	579
354/419-07	yes	1	22341	23407
354/419-11	n.d	n.d.	1077	215
354/419-12	n.d.	n.d.	n.d.	n.d.
354/419-13	yes	9	736	346
354/419-15	yes	2	208	208
354/419-18	yes	7	230	62
354/419-21	yes	3	186	56

n.d = not determined

Rice plants regenerated from transgenic callus stained positively for GUS in both roots and leaves indicating constitutive expression. It was not expected that constitutive expression of GUS would be observed from the pDAB419 construct because of the lack of expression in the leaves of the native *per5* gene in maize.

Example 10

Transformation of Maize with pDAB 419

A. Establishment of Type II Callus Targets.

Two parents of 'High II' (Armstrong and Phillips, (1991)) were crossed and when the developing embryos reached a size of 1.0-3.0 mm (10-14 days after pollination), the ear was excised and surface sterilized. Briefly, ears were washed with Liquinox soap (Alconox, Inc., NY) and subjected to immersions in 70% ethanol for 2-5 minutes and 20% commercial

bleach (0.1% sodium hypochlorite) for 30-45 minutes followed by 3 rinses in sterile, distilled water. Immature embryos were isolated and used to produce Type II callus.

For Type II callus production, immature embryos were placed (scutellum-side up) onto the surface of 'initiation' medium (15Ag10) which included N6 basal salts and vitamins (Chu, 1978), 20 g/L sucrose, 2.9 g/L L-proline, 100 mg/L enzymatic casein hydrolysate (ECH), 37 mg/L Fe-EDTA, 10 mg/L silver nitrate, 1 mg/L 2,4-dichloro-phenoxyacetic acid (2,4-D), and 2.5 g/L Gelrite (Schweizerhall, NJ) with pH adjusted to 5.8. After 2-3 weeks incubation in the dark at 28°C, soft, friable callus with numerous globular and elongated somatic embryo-like structures (Type II) were selected. After 2-3 subcultures on the 'initiation' medium, callus was transferred to 'maintenance' medium (#4). The 'maintenance' medium differed from the 'initiation' medium in that it contained 690 mg/L L-proline and no silver nitrate. Type II callus was used for transformation experiments after about 16-20 weeks.

B. Helium Blasting and Selection.

pDAB367 (Example 27) and pDAB419 were co-precipitated onto the surface of 1.5-3.0 micron gold particles (Aldrich Chem. Co., Milwaukee, WI). pDAB367 contains a phosphinothricin acetyl transferase gene fusion which encodes resistance to the herbicide Basta.™ This gene is used to select stable transgenic events. The precipitation mixture included 60 mg of pre-washed gold particles, 140 µg of plasmid DNA (70 µg of each) in 300 µL of sterile water, 74 µL of 2.5 M $CaCl_2$, and 30 µL of 0.1 M spermidine. After adding the components in the above order, the mixture was vortexed immediately, and allowed to settle for 2-3 minutes. The supernatant was removed and discarded and the plasmid/gold particles were resuspended in 1 mL of 100% ethanol and diluted to 7.5 mg plasmid/gold particles per mL of ethanol just prior to blasting.

Approximately 400-600 mg of Type II callus was placed onto the surface of #4 medium with 36.4 g/L sorbitol and 36.4 g/L M mannitol for 4 hours. In preparation for blasting, the callus was transferred to #4 medium with 2% agar (JRH Biosciences, Lenexa, KS) and covered with a stainless steel screen (104 micron). Helium blasting was completed using the same device described in Example 9. Each callus sample was blasted a total of four times. After blasting the callus was returned to #4 medium with 36.4 g/L sorbitol and 36.4 g/L mannitol for 18-24 hours after which it was transferred to 'selection' medium (#4 medium with 30 mg/L Basta™ and no ECH or L-proline). The callus was transferred to

fresh 'selection' medium every four weeks for about three months. After 8-12 weeks, actively growing transgenic colonies were isolated and sub-cultured every two weeks on fresh 'selection' medium to bulk-up callus for regeneration.

C. Histochemical GUS Assay.

Basta™-resistant callus was analyzed for GUS expression by incubating a 50 mg sample in 150 µL of assay buffer for 48 hours at 37°C. The assay buffer consisted of 0.2 M sodium phosphate pH 8.0, 0.5 mM each of potassium ferricyanide and potassium ferrocyanide, 10 mM sodium EDTA, 1.9 mM 5-bromo-4-chloro-3-indolyl-b-D-glucuronide, and 0.06% v/v Triton x-100 (Jefferson *et al.*, 1987). Transgenic callus expressing the GUS gene turned blue. A total of 17 Basta™-resistant callus lines were established for maize, with three maize lines expressing the GUS gene as evidenced by blue staining after 48 hours in the GUS histochemical assay.

Table 6. Histochemical GUS Staining of Transgenic Maize Callus

Line	rating
311/419-01	+
311/419-02	++
311/419-16	+++

+ = Occasional blue region
++ = Light blue staining throughout
+++ = Dark blue regions
++++ = Intense blue staining throughout

There was considerable variability in intensity of staining among the expressing callus ranging from very intense to somewhat spotty (Table 6). Generally, callus staining was more intense in rice than in maize.

D. Plant Regeneration.

GUS-expressing callus was transferred to 'induction' medium and incubated at 28°C, 16/8 light/dark photoperiod in low light (13 mE/m^2/sec) for one week followed by one week in high light (40 mE/m^2/sec) provided by cool white fluorescent lamps. The 'induction' medium was composed of MS salts and vitamins (Murashige and Skoog (1962)), 30 g/L sucrose, 100 mg/L myo-inositol, 5 mg/L 6-benzylamino purine, 0.025 mg/L 2,4-D, 2.5 g/L Gelrite (Schweizerhall, NJ) adjusted to pH 5.7. Following this two-week induction period, the callus was transferred to 'regeneration' medium and incubated in high light (40

mE/m^2/sec) at 28°C. The 'regeneration' medium was composed of MS salts and vitamins, 30 g/L sucrose, and 2.5 g/L Gelrite (Schweizerhall, NJ) adjusted to pH 5.7. The callus was sub-cultured to fresh 'regeneration' medium every two weeks until plantlets appeared. Both 'induction' and 'regeneration' medium contained 30 mg/L Basta™. Plantlets were transferred to 10 cm pots containing approximately 0.1 kg of dry Metro-Mix (The Scotts Company, Marysville, OH), moistened thoroughly, and covered with clear plastic cups for approximately 4 days. At the 3-5 leaf stage, plants were transplanted to 5-gallon pots and grown to maturity.

E. Southern Analysis

A DNA probe specific for the β-glucuronidase (GUS) coding region was excised from the pDAB418 plasmid using the restriction enzymes *Nco*I and *Sst*I. The 1.9 kb fragment was purified with the Qiaex II DNA purification kit (Qiagen Inc., Chatsworth, CA). The probe was prepared using an oligo-labeling kit (Pharmacia LKB, Piscataway, NJ) with 50 microcuries of a^{32}P-dCTP (Amersham Life Science, Arlington Heights, IL). Southern analysis was used to identify maize callus material that contained an intact copy of the transgene and to measure the complexity of the integration event. The callus material was removed from the media, soaked in distilled water for 30 minutes and transferred to a new petri dish, prior to lyophilization. Genomic DNA from the callus was prepared from lyophilized tissue as described by Saghai-Maroof *et al.* (1984). Eight micrograms of each DNA was digested with the restriction enzyme *Xba*I using conditions suggested by the manufacturer (Bethesda Research Laboratory, Gaithersburg, MD) and separated by agarose gel electrophoresis. The DNA was blotted onto nylon membrane as described by Southern (1975, 1980). The GUS probe was hybridized to the genomic DNA on the blots. The blots were washed at 60°C in 0.25X SSC and 0.2% SDS for 45 minutes, blotted dry and exposed to XAR-5 film overnight with two intensifying screens.

F. Screening of R_0 Plants for Uniform Expression.

The 6th leaf was collected from five or six "V6-equivalent" stage plants (because of inability of determining exact leaf number from R0 plants, a plant characteristic of the V6 stage was used). The entire leaf was removed, cut into pieces and stored in a plastic bag at -70°C until further processing. Leaves were powdered in liquid nitrogen and tissues samples representing approximately 400 µL of tissue were placed in microfuge tubes. The tissue was

either stored or extracted immediately. GUS was extracted by mixing the powdered tissue with GUS Lysis Buffer (Jefferson, 1987) as modified by the addition of 1% polyvinylpyrrolidone (hydrated in the buffer for at least one hour), 20% glycerol, 50 mg/mL antipain, 50 mg/mL leupeptin, 0.1 mM chymostatin, 5 mg/mL pepstatin and 0.24 mg/mL Pefabloc™ (Boehringer Mannheim, Indianapolis, IN). After incubation on ice for at least 10 min, the samples were centrifuged at 16,000g for 10 min. The supernatants were recovered and centrifuged a second time as described above. The supernatants were recovered and frozen on dry ice and stored at -70°C. Experiments showed that GUS activity was stable for at least 4 freeze-thaw cycles when stored in the buffer described above. GUS activity was measured using a GUS-Light™ kit (Tropix, Inc, Bedford, MA). Five µL samples of undiluted extract or of extract diluted so that the luminescence was within the range measured by the luminometer was added to 195 µl of the GUS-Light™ Reaction Buffer. After 1 hr the luminescence was measured using a BioOrbit 1251 luminometer equipped with a BioOrbit 1291 injector after injection of 300 µL of GUS-Light™ Accelerator. Luminescence was integrated for 5 sec after a 5 sec delay. Protein was measured with the assay developed by Bradford (1976) using human serum albumin as the standard.

G. Organ-Specific Expression Quantitative Analyses.

Plants grown in the greenhouse in 5 gallon pots were harvested to determine organ-specificity of GUS expression. Prior to harvesting tissue from V6-equivalent plants, roots were cut approximately one inch from the side of the pot to remove any dead root tissue. Roots from VT stage (mature) plants were washed and any dead root tissue was removed before freezing at -70°C. Leaves, stems (VT-stage plants only) and roots were harvested and either frozen at -70°C or powdered in liquid nitrogen immediately. Experiments showed that GUS is stable in frozen tissue. After powdering the tissues, three aliquots of approximately 10 ml of tissue were collected into preweighed tubes, and the tubes with tissue weighed and stored at -70°C. Tissue was extracted in the same buffer as described above except protease inhibitors were only added to aliquots of the extracts instead to the entire extract volume. For extraction, the powdered tissues were thawed into 4 ml buffer/g tissue and homogenized for 5-10 sec at 8,000 rpm using a Ultra-Turrax T 25 (IKA-Works, Inc.) homogenizer with an 18 mm probe. The samples were centrifuged at 4°C for 5 min at 2015g. After removing the supernatants, the pellets were extracted again but with 2 ml

buffer/g tissue and the supernatant after centrifugation was pooled with the supernatant from the first extraction. The pellet was extracted again with 2 ml/g tissue; the supernatant after centrifugation was processed separately from the pooled supernatants from the first two extractions. GUS activity recovered in the final extract was used to determine extraction efficiency of the first two extractions. GUS and protein assays were done as described above for both sets of supernatants. Roots at each node from V7 plants grown in approximately 15 gallon pots were analyzed separately as described above.

H. Histochemical Analyses Staining of Maize Tissues.

Histochemical analyses of per5*adh*/GUS/*nos* gene expression was done essentially as described by Jefferson (1987). Roots were first treated 1 h at 37°C in 100 mM $NaPO_4$ buffer, pH 7.0, 10 mM EDTA, 0.1% Triton X-100 and 10 mM ß-mercaptoethanol. The root sections were washed 3 times with the same buffer but without ß-mercaptoethanol and then incubated 1hr in the same buffer at 37°C. GUS histochemical assay buffer Jefferson (1987) was added and the tissues were incubated for various times at 37°C. Roots from V6 and VT plants were removed from each node and treated separately. Roots from each node of V6 plants were measured, cut into 6 equal parts, and 2-one centimeter pieces were removed from the ends of each root section. One root piece from each section was stained until the ends were blue; the other piece from each section was stained overnight. Roots from VT plants were stained similarly, but two roots from each node, if available, were cut into several pieces and stained together. One root from each node was stained until the roots turned blue; the other root from each node was stained overnight. One intact leaf was removed from the bottom, middle and top of the V6 and VT plants and analyzed. The leaves were cut lengthwise. The leaf half containing the midrib was transversely cut at intervals across the midrib and along the outer edge of the leaves. The leaves were vacuum infiltrated with GUS histochemical assay buffer and incubated at 37°C until stained regions were visible. Chlorophyll was removed by incubation in 70% ethanol at room temperature. Pieces of stems that included a node and adjacent internodal regions were cut from the bottom, middle and top sections of VT plants. Cross sections of the internodal regions and longitudinal sections that included the node and internodal regions above and below the node were stained. One longitudinal and one cross sectional piece of each stem region analyzed was stained until blue was visible; another set of stem pieces was stained overnight. After

staining, the stem pieces were placed in 70% alcohol to remove chlorophyll. Pollen was collected from transgenic per5*adh*/GUS/*nos* plants for 2 hr from tassels from which all extruded anthers were removed. Pollen was stained overnight. Kernels were analyzed 20 days post-pollination from crosses done in which the transgenic plant was the male parent and from crosses in which the transgenic plant was the female parent. The kernels were dissected longitudinally through the embryo.

I. Screening of R_0 Plants for Uniform Expression.

To define the spatial and temporal expression patterns of a promoter of interest, the expression pattern of a transgene must not be affected by its chromosomal location. Evidence suggests that transgene expression can be "silenced" non-uniformly in different parts of plants, resulting in spatial and temporal expression patterns that do not represent the true promoter activity in transgenic plants. Gene silencing often occurs stochastically, occurring to different extents in individuals within a population (reviewed by Matzke *et al.* (1993)). All transformation events were screened for uniform expression among five or six R_0 plants for each event (Table 7), thus eliminating transformation events that display silencing of the transgene in a population of this size. GUS expression among R_0 plants analyzed for each of three transformation events reported here were statistically indistinguishable.

Table.7 Expression of GUS with pDAB 419 in Individual R_0 Plants in Three Transformation Events

TRANSFORMATION EVENTS						
308/419-01[a]			419-02		419-16	
Relative Light Units/mg Protein	*Standard Deviation*[b]		*Relative Light Units/mg Protein*	*Standard Deviation*[b]	*Relative Light Units/mg Protein*	*Standard Deviation*[b]
24973	853		5261	562	1011	97
23811	641		4537	381	1039	14
29747			5055	573	1213	9
24081	614		5743	137	942	12
25729	199		4645	315	1367	57
27025					1282	46

[a]only one sample was analyzed for some of the 308/419-01 plants

[b]standard deviations were determined from independent analyses of two aliquots of tissue from each plant

J. Quantitative Analyses of pDAB 419 Maize Plants.

Quantitative analyses of GUS activity was done at two stages of corn development: V6 (whorl stage) and VT (tassel emergence). Entire leaf, stem or root samples were powdered and duplicate aliquots were analyzed. GUS activity was determined relative to either extracted protein concentration or to fresh weight of tissue. The high percent recovery of GUS activity indicates extraction procedure for GUS is efficient (Tables 8 and 9). The 308/419-01 and 419-02 plants are BC_1 (crossed consecutively with the same inbred twice) and R_0 generations, respectively. The per5*adh* promoter is expressed in root, stem (VT plants) and leaf tissue (Tables 8 and 9). When normalized to extractable protein, roots express higher levels of GUS than leaves in V6 and VT plants; stem accumulates GUS at levels higher than either leaves or roots in VT plants (Tables 8 and 9). GUS expression normalized to fresh weight of tissue and expression normalized to extractable protein levels follow similar trends of organ-specificity of expression in VT plants, although the relative proportions of expression among the organs are different. In V6 plants, the per5*adh* promoter expresses GUS at similar levels in leaves and roots based on fresh weight of tissue, but the promoter clearly expresses GUS higher in roots than in leaves when expression is normalized to extractable protein.

Table 8. Expression of Per5adh/GUS/*nos* in V6 Transgenic Plant Organs

Plant Organ	Relative Light Units/mg Protein	Standard Deviation[a]	Relative Light Units/g Tissue (÷1000)	Standard Deviation[a]	Average Percent Extraction Efficiency[b]
308/419-02					
leaves	5,518	155	39,687	4,231	86.8
roots	15,496	2,918	33,155	7,620	91.1
419-02					
leaves	3,256	111	23,367	1,704	85.8
roots	8,871	35	14,316	333	89.3

[a]standard deviations were determined from independent analyses of two aliquots of tissue from each sample

[b]extraction efficiency was percent recovery of GUS activity in the first two extractions relative to the total GUS activity in all three extractions of the tissues

Table 9. Expression of Per5adh/GUS/*nos* in VT Transgenic Plant Organs

Plant Organ	Relative Light Units/mg Protein	Standard Deviation[a]	Relative Light Units/g Tissue (÷1000)	Standard Deviation[a]	Average Percent Extraction Efficiency[b]
308/419-02					
leaves	2,915	177	30,426	1,567	87.3
stem	15,701	837	35,601	593	85.2
roots	10,197	351	15,393	310	82.8
419-02					
leaves	2,319	15	18,112	1,305	86.7
stem	14,721	165	32,619	747	84.0
roots	3,923	734	6,473	814	83.1

[a]standard deviations were determined from independent analyses of two aliquots of tissue from each sample

[b]extraction efficiency was percent recovery of GUS activity in the first two extractions relative to the total GUS activity in all three extractions of the tissues

The *per5adh* promoter activity was examined in detail in roots. For these experiments, 308/419-01 plants were grown in 15 gallon pots to improve root quality. Roots at all nodes express GUS, but the GUS activity/mg extractable protein increases in nodes 3-5 relative to expression in nodes 1 and 2 (Table 10).

Table 10. Expression of GUS with pDAB 419 in Transgenic Plant Root Nodes

Root Node	Relative Light Units/mg Protein	Standard Deviation[a]
node 1	5,479	
node 2	4,268	297.5
node 3	6,836	47.3
node 4	8,148	92.6
node 5	10,887	305.9

[a]standard deviations were determined from independent analyses of two aliquots of tissue from each sample; only one sample was available for node 1

K. Histochemical Analyses of pDAB 419 Maize Plants.

The per5*adh* promoter expresses GUS to levels that are detectable in all tissues tested using the histochemical staining procedure of Jefferson (1987) with the exception of kernels (but only when the transgenic plant is used as a pollen donor) and pollen. Roots at all nodes of these transgenic plants express GUS. GUS is expressed over the entire length of the roots with the exception that in at least some roots, the expression drops dramatically at

the distal end of the root. The loss of stainable activity in the root ends is not due to technological limitations of the protocol in that roots from transformation events expressing transgenes driven by other promoters express highly in these regions. The stem stains for GUS activity non-uniformly, with the pith showing poor or no staining; the nodes and areas adjacent to the outer edge of the stem stain. Most of the areas that stain correspond to regions rich in vascular tissue. The blade, sheath and the midrib of the leaves express GUS. Kernels do not display any stainable activity in overnight incubations in GUS histochemical staining solution when the kernels are from crosses using the per5*adh*/GUS/*nos* plants as the pollen donor. However, when the transgenic plant is used as the maternal parent in the cross, GUS is expressed in the pericarp (seed coat) as well as a discrete area of the embryo.

Expression patterns of maize plants transformed with pDAB419 were similar to the expression patterns observed in transgenic rice. The *per5* promoter/*adh I* intron combination appear to promote a pattern of expression which is constitutive. That is, significant expression is observed in both roots and leaves. This is unexpected as the *per 5* gene is natively root-preferentially expressed. This result is consistent with the expression pattern that was observed in rice.

Example 11

PerGUS 16

PerGUS 16 is a plasmid containing 4kb of *per5* promoter, the *per5* untranslated leader sequence, the coding sequence for the first five amino acids of *per5*, the GUS gene, and the *nos* 3'UTR. The complete sequence of PerGUS 16 is given in SEQ ID NO 15. With reference to SEQ ID NO 15, significant features of PerGUS16 are given in Table 11.

Table 11: Significant Features of PerGUS 16

nt (SEQ ID NO 15)	Features
1-6	SstI site
37-42	BamHI site
43-48	SalI site
48-53	NcoI site
48-4247	*Per5* promoter nt 1-4200 of SEQ ID NO 1 and untranslated leader
4248-4263	*Per5* exon nt 4201-4215 of SEQ ID NO 1
4264-6068	β glucuronidase gene (GUS)
6069-6111	untranslated sequence from pBI221
6122-2127	SstI site
6122-6396	*nos* 3' UTR
6397-6407	linker
6402-6407	HindIII site
6408-9299	Bluescript ® II SK$^-$

PerGUS16 is different from pDAB411 in that PerGUS16 includes the coding sequence for the first 5 amino acids of the *per5* protein. In addition PerGUS16 contains 4 kB of upstream promoter sequence, whereas pDAB411 only contains 2 kB of sequence. Neither PerGUS 16 nor pDAB411 includes an intron in the untranslated leader. PerGUS16 was constructed and tested in a transient maize root expression assay as follows.

A. Construction of PerGUS 16. A 4.0 kB *Nco*I fragment, containing 4 kB of upstream *per5* sequence, the *per5* untranslated leader sequence and the coding sequence for the first 5 amino acids of *per5*, from perGEN1(10.4) was purified from a 1.0% agarose gel using Qiagen kit. This 4.0 kB promoter fragment was ligated into an *Nco*I site at the translation initation start site of the GUS gene in pGUS*nos*12. pGUS*nos*12 is a plasmid based on Bluescript ® II SK$^-$ with an inserted *Bam*HI-*Hin*dIII fragment containing the coding region for the GUS gene and the *nos* 3' UTR. The resultant translation fusion is PerGUS16.

B. Expression Assay. Results of testing PerGUS16 in a transient maize root expression assay are given in Table 14.

Example 12

PERGUSPER3

PERGUSPER3 is a plasmid containing 4kb of *per5* promoter, the *per5* untranslated leader sequence, the coding sequence for the first five amino acids of per5, the GUS gene,

and the *per5* 3' UTR. The complete sequence of PERGUSPER3 is given in SEQ ID NO 16. With reference to SEQ ID NO 16. critical features of PERGUSPER3 are as follows:

Table 12: Significant Features of PERGUSPER3

nt (SEQ ID NO 16)	Features
1-6	SstI site
1-42	Bluescript SK polylinker
37-42	BamHI site
43-48	XbaI site
43-53	synthetic linker
54-59	NcoI site
54-4253	Per5 promoter nt 1-4200 SEQ ID NO 1
4254-4269	Per 5 exon nt 4201-4215 SEQ ID NO 1
4264-4269	NcoI site
4266-6074	β glucuronidase gene (GUS)
6075-6117	untranslated sequence from pB1221
6135-6140	XhoI site
6140-6510	Per5 3' UTR nt 6069-6439 SEQ ID NO 1
6511-6516	HindIII site
6517-9408	Bluescript® II SK⁻

PERGUSPER3 is identical to PerGUS 16 except for its 3' UTR. PerGUS16 has the *nos* and PERGUSPER3 has the *per5* 3' UTR. Neither PERGUSPER3 nor PerGUS 16 has an intron in the untranslated leader. PERGUSPER3 was constructed and tested in a transient maize root assay, in stable transformed rice callus, and in stable transformed rice plants as follows.

A. Construction of PERGUSPER3

1. BSGUSper4. The 3' UTR from the *per5* gene was amplified on a 396 bp fragment (corresponding to bp 6069 to 6439 of SEQ ID NO 1 plus 26 bases of synthetic linker sequence) from the plasmid perGEN1(10.4) using Amplitaq polymerase with buffers supplied and synthetic primers,

TTATCTCGAGGGCACTGAAGTCGCTTGATGTGCTGAATT (SEQ ID NO 17) and GGGGAAGCTTCTCTAGATTTGGATATATGCCGTGAACAATTG (SEQ ID NO 18). The 5' primer added an *Xho*I restriction site, and the 3' primer included a *Hin*dIII site, to facilitate cloning. This fragment contains a canonical AAUAAA poly-A addition signal at position 247 (corresponding to bp 6306 of SEQ ID NO 1). The amplification product was ligated into an *Xho*I/*Hin*dIII of plasmid pDAB356/X [Note: The structure of plasmid pDAB356/X is not directly relevant to the end result of this construction series. It was constructed during an unrelated series, and was chosen because it contained restriction

recognition sites for *Xho*I and *Hin*dIII at the 3' end of the GUS coding region. Those skilled in the art will realize that other plasmids can be substituted at this step with equivalent results.] and transformed into DH5α. Ampicillin resistant transformants were screened by colony hybridization using the *per5* 3' UTR amplification product as a probe.

Three of the resulting transformants hybridized to ^{32}P radiolabelled 3' UTR amplification product. The plasmid from each of these three transformants was extracted for sequence analysis. Sequence analysis using an Applied Biosystems automated sequencer revealed that a clone designated p3'per26 was free of PCR induced errors. A 2.0 kB *Bam*HI/*Hin*dIII fragment from p3'per26 containing the GUS-*per5* 3' UTR was gel purified as described above and ligated into the BamHI/HindIII cloning site of Bluescript ® II SK⁻. One of the resulting plasmids, designated BSGUSper4, was characterized and selected for subcloning.

2. PERGUSPER3. The 4.0 kB *Nco*I *per5* promoter fragment from perGEN1(10.4) described above was ligated into the *Nco*I site of BSGUSper4 (the translational initiation of the GUS gene). The resultant clone, PERGUSPER3, contains 4 kB of *per5* promoter, the *per5* untranslated leader sequence, the first 5 amino acids of *per5*, the GUS gene, and the *per5* 3' UTR.

B. Expression Assays. Results of testing PERGUSPER3 in a transient maize root assay are given in Table 14. Results of testing PERGUSPER3 in stable transformed rice callus and rice plants is given in Tables 15.

Example 13
5' Deletions of PERGUSPER3

A series of 5' deletions of PERGUSPER3 was assembled to test the effect on expression. Construction of these vectors utilized naturally occurring restrictions sites in the 4.0 kB *Nco*I promoter region.

A. Construction of SPGP1

SPGP1 is identical toPERGUSPER3 except for the absence of 2 kB of 5' upstream sequence (i.e., bp 25 to 2585 of SEQ ID NO 16 are deleted). SPGP1 was derived from PERGUSPER3 by subcloning the *Xba*I fragment of PERGUSPER3 into the XbaI site of Bluescript ® II SK⁻

B. Construction of HSPGP4.

HSPGP4 is identical to SPGP1 except for the absence of 1 kB of 5' upstream sequence (i.e., bp 25 to 3240 of SEQ ID NO 16 are deleted). This vector was derived from SPSP1 by the deletion of the 1 kB *Hind*III fragment.

C. Construction of PSPGP1

PSPGP1 is identical to SPGP1 except for the absence of 1.9 kB of PstI sequence (i.e., bp 25 to 4139 of SEQ ID NO 16 are deleted). PSPGP1 only had 109 bases of 5' sequence which includes the TATA box.

D. Expression Assay. Results of testing SPGP1, HSPGP4 and PSPGP1 in a transient maize root expression assay are given in Table 14.

Example 14

Transient Root Expression Assay

Transient assays have been successfully used for studying gene expression in plants, especially where an efficient stable transformation system is not available (ie., maize, wheat). In protoplasts, these assays have been used to study the expression of regulatory elements with relatively simple expression patterns. For example, constitutive promoters, including the CaMV 35S, have been extensively studied in maize protoplasts. Luehrsen and Walbot (1991). However, it was believed that a root preferrential promoter, such as *per5*, would be unlikely to function normally in protoplasts, particularly those derived from tissue culture. Therefore, a system to study expression in intact root tissue was desirable. Particle bombardment of root tissue would enable transient expression analysis and reduce the need for production of stable transgenics.

A. Helium Blasting into Roots. Captan™-treated seed of CQ806 and OQ403 were soaked for 45 min., rinsed 3 times in sterile distilled water, and germinated in sterile petri dishes (100x25 mm) containing Whatman #1 filter paper moistened with sterile milli Q water for about 4-7 days. Approximately 1 cm size root tips were excised and arranged (6 per target) in 'blasting' medium (#4 with 2% agar). The 'blasting medium' consisted of N6 basal salts and vitamins (Chu, 1978), Fe-EDTA, 20 g/L sucrose, 690 mg/L L-proline, 100 mg/L enzymatic casein hydrolysate (ECH), 1 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), and 20 g/L agar. The roots were covered with a 204 micron screen prior to blasting. Each target was blasted once at 1,500-2,000 psi using two times dilution of gold/DNA solution.

The gold particles (Biorad 1.0 micron) were coated with DNA (different plasmids as mentioned in the text) as described in Example 10B. Different blasting parameters, i.e., 1) different helium pressures (500, 1,000, 1,500, and 2,000 psi), 2) number of blastings per target (1-4 blastings per target), 3) concentration of gold/DNA (1-4 times dilutions of gold/DNA solution), 4) particle size (Aldrich 1.5-3.0 micron vs. Biorad 1.0 micron gold particles), and 5) high osmoticum treatment (0.2M mannitol and 0.2M sorbitol treatment 4h prior to and 16-18 h after blasting) were tested. Following blasting, roots were transferred to 15Ag10-2D medium and incubated in the dark at 27° C. The 15Ag10-2D medium differed from #4 medium in that it contained 2.9 g/L L-proline, 10mg/L silver nitrate, 2 mg/L 2,4-D, and 2.5 g/L Gelrite.

B. Histochemical GUS Assay After 18-24 hrs , the blasted roots were assayed for transient GUS expression according to Jefferson (1987). Roots were placed in 24-well microtitre plates (Corning, New York, NY) containing 500 µL of assay buffer per well (six per well). The assay buffer consisted of 0.1 M sodium phosphate (pH 8.0), 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 10 M sodium EDTA, 1.9 mM 5-bromo-4-chloro-3-indolyl-beta-D-glucuronide, and 0.06% triton X-100. The plates were incubated in the dark for 1-2 days at 37° C before observations of GUS expression under a microscope.

C. Optimization of DNA Delivery into Roots. Transient expression increased with increased helium pressure with highest levels observed at 1,500-2,000 psi. High osmoticum treatment prior to blasting did not enhance GUS expression. Also, increasing the number of blastings per target did not result in increased expression. One blasting per target yielded highest expression in roots of both OQ403 and CQ806. In addition, two times dilution of gold/DNA solution and use of the Biorad 1.0 micron particles were found to be most suited for obtaining consistently high levels of expression. Based on these results, a set of conditions were established for blasting into roots. With these conditions, 60-100% of the blasted roots expressed GUS with an average number of ca. 50 GUS expression units per target using pDAB418 (Ub1-GUS-*nos*).

D. Transient Expression of Different *per5* Constructs in Roots. Transient GUS expression of different *per5* constructs was tested in roots following helium blasting using the conditions described above. The results from ten different experiments are summarized in Table 14.

TABLE 14. Transient expression of different *per5* constructs in roots.

Plasmid	Description	# GEUs*	(N)‡	Rating
PerGUS16	4.5 kB *per5*, first 5 aa of *per5* protein-GUS-*nos*	3.4	(24)	++
PERGUSPER3	4.5 kB *per5*, first 5 aa of *per5* protein-GUS-*per5*	10.0	(24)	++++
SPGP1	2.0 kB *per5*, first 5 aa of *per5* protein-GUS-*per5*	10.7	(24)	++++
HSPGP	1.0 kB *per5*, first 5 aa of *per5* protein-GUS-*per5*	5.8	(15)	+++
PSPGP	0.1 kB *per5*, first 5 aa of *per5* protein-GUS-*per5*	10.8	(16)	++++
pDAB411	2.0 kB *per5*-GUS-*nos*	1.1	(5)	+
pDAB419	2.0 kB *per5*, *Adh1* intron1-GUS-*nos*	6.7	(3)	+++

* GUS expression units (number of blue spots observed) per target

‡ N= # of targets blasted

pDAB411, the construct containing 2.0 kB *per5*, expressed at very low levels. With PerGUS16 containing 4.0 kB *per5* and a fusion including the first five amino acids of the *per5* protein, the expression was 3-fold higher than that of pDAB411. Further, PerGUSper3 consisting of *per5* with the 3' UTR showed a further 3-fold increase over PerGUS16 demonstrating that 3' end is also important for regulation of expression. Although SPGP1 contained 2.0 kB of *per5*, no difference was observed between the expression of SPGP1 and PerGUSper3. With additional deletion in the 5' region of *per5* in HSPGP (which contains 1.0 kB of *per5*), expression was decreased over that of SPGP1 and PerGUSper3. However, relatively high levels of expression were observed with PSPGP containing only 0.1 kB region of *per5*.

Probably all of the promoter elements which were necessary for maximal root specific expression are present in the first 1 kB of 5' sequence. However, elements which may suppress expression in other tissues may not be present in this 1 kB sequence. Similar observations have been made with the 5' upstream sequences of the *Sus4* gene from potato which contains a negative element that suppresses expression in stems and leaves. Fu *et al.* (1995). Transient assays in other tissues would be necessary to obtain this information from the *per5* constructs. Expression from PSPGP, which contained only 100 bases 5' sequence, probably acts as a basal promoter and, therefore, would not be expected to contain the elements necessary for root specific expression nor enhancer elements necessary for maximal activity of the promoter. Expression from this construct in stable plants would be expected to be constitutive.

A translational fusion of the *per5* gene which included the *per5* 5' untranslated leader (UTL) and the first 5 amino acids of the *per5* gene fused to the *uidA* was included in PerGUS16, PERGUSPER3, SPGP1, HSPGP, and PSPGP constructs. The ability of these

constructs to express GUS, demonstrated that this UTL sequence was capable of promoting translation and therefore can be used to express commercially important transgenes.

The most obvious improvement in expression was observed from the addition of the *per5* 3' UTR in place of the *nos* sequence. 3' UTR's are known to contain sequences which affect gene expression by altering message stability (Sullivan and Green (1993)) or influencing translation (Jackson and Standart (1990)). Examples include polyadenylation signals (Rothnie *et al.* (1994)) and destabilizing elements (Gallie *et al.* (1989)). However, the *per5* and *nos* 3'UTR's cannot be distinguished by the presence or absence of these sequences. Both UTR's contain a canonical AAUAAA poly-A addition signal. Neither sequence appears to contain any of the published destabilizing elements. An obvious difference between the two UTR's is the length; the longer *per5* UTR may confer greater stability of the message.

Example 15
Rice Transformation of PERGUSPER3
Transgenic Production and Histochemical GUS Assay

To study the expression of PerGUSPer3 in transgenic rice, a total of 35 independent transgenic lines were produced. Out of these, plants of 9 lines (354/PERGUSPER3-03,20,21,23,24,27,28,30,and 34) displayed GUS expression in roots. Although GUS expression was variable from line to line, a few lines showed very intense expression in roots. Histochemical GUS analysis of different tissues following vacuum infiltration showed GUS expression in cut portions of leaves, glumes, anthers, pollen and embryo. No expression was seen in endosperm. All of these results suggest that *per5* expresses in a constitutive manner in rice.

Rice plants from six PERGUSPER3 Ro lines were characterized by Southern analysis. The rice DNA was also cut with the restriction enzyme *Xba*I which should result in a 4.2 kb fragment when hybridized to the GUS probe. All of the six lines contain the gene construct. A moderately complex integration event was detected in one of the six lines containing an intact copy of the gene construct. The remaining five lines all had complex integration events with as many as nine hybridization products. A summary of the genetic analysis is located in Table 15.

Table 15: Assay of Transformed Rice Plants

Plant	Presence of the Intact Gene Construct	Number of Hybridization Products	Gus Histochemical Results	Relative Light Units per ug of protein - Root	Relative Light Units per ug of protein - Leaf
354/PGP3-20	Yes	5	Positive	13,129	26,220
354/PGP3-21	Yes	9	Positive	1,579	623
354/PGP3-22	n.d.	-	Negative	5	11
354/PGP3-23	Yes	4	Positive	61	20
354/PGP3-24	Yes	3	Positive	1,484	1,398
354/PGP3-27	Yes	6	Positive	115	12
354/PGP3-28	Yes	5	Positive	338	222

n.d. - not determined

Both longitudinal and transverse root sections prepared from transgenic rice seedlings showed cells with GUS expression (blue color) and cells interpreted to lack GUS expression (red color resulting from the counterstain). Longitudinal section of a primary root showed GUS expression present in all cells except for those present in the root cap, meristematic zone, and a portion of the cell elongation zone. This pattern of expression was confirmed for secondary root formation in a transverse section of root tissue. Cross section of a primary root, prepared from within the zones of cell elongation and differentiation, showed most cells expressing GUS. Very intense GUS expression (dark blue) was observed in the exodermis or outer cortex of the root sample. GUS expression was noted as slight to absent in the epidermal layer even though root hairs were observed macroscopically to be blue. Both vascular and cortical tissues showed moderate expression. Based on the consistent staining patterns obtained from free hand tissue sections, cells in the vascular and cortical tissues genuinely expressed the GUS protein rather than appear as artifacts with the diffusion of histochemical stain from the exodermis.

Analysis of variance showed that sample to sample variation within each of the independent events was not significant. However, most of the variation was associated among the different events. Based on the GUS quantitative data, only event 354/PERGUSPER3-20 was shown to be highly significant different ($p<0.001$) from zero (Table 15) even though five other events were shown to be histochemically GUS positive.

The maize *per5* 5' region in combination with the 3' untranslated sequences promoted high-level expression of the introduced β-glucuronidase gene in young transgenic rice plants.

Functional activity was observed in both roots and leaves. Quantitative data indicated that there was considerable variability of expression between the different events. This variability is most likely a result of a combination of factors including position effects of the integrated transgene, differences in copy number of the insertion products, and rearrangements of the insertion events. All of these variables have the potential to effect expression levels and have been documented in most transgenic studies.

Despite high degree of variability in the expression levels, the expression pattern of PerGUSPer3 in different transformation events was consistent. Slight to very intense expression was evident in the entire primary and secondary roots except in the root tips. Histological analysis showed very intense expression in the outer cortex and moderate expression in cortex and vascular tissues. Such pattern and level of expression observed appears to be very suitable for expression of genes to control root pests (i.e., root weevil). In addition, consistent with expression in roots, high levels of expression was also observed in stem and leaf tissue (quantitative data) thus providing opportunity for controlling other insects (i.e., stem borer). These data demonstrate that the *per5* promoter, in the absence of an intron, drives constitutive expression of transgenes in rice.

Example 16

Maize Transformation of PERGUSPER3

Establishment of typeII callus targets and helium blasting conditions were that same as described in Example 10. A total of 82 independent transgenic colonies of maize were produced. Of these, 55 lines were subjected to Southern analysis as described in Example 15. Twenty-nine lines were found to be Southern positive and contained an intact hybridization product of the GUS gene. Following GUS histochemical assay, callus of about 72 lines showed no expression. Also, roots and leaves of different Southern-positive lines displayed no GUS expression when callus was regenerated on the 'regeneration' medium. This data supported the observation that sequences other than the 5' promoter region and the 3' UTR were critical for expression in corn.

Example 17

Plasmid PIGP/367

Plasmid PIGP/367 contains the *per5* promoter, the *per5* untranslated leader modified to include the *per5* intron 1, the GUS gene, and the *per5* 3'UTR. The complete sequence for PIGP/367 is given in SEQ ID NO 19. With reference to SEQ ID NO 19, critical features of PIGP/367 are given in Table 16.

Table 16: Significant Features of PIGP/367

nt (SEQ ID NO 19)	Features
1-40	synthetic polylinker
41-75	pCR™2.1 polylinker
81-1741	*Per5* promoter nt 2532-4192 SEQ ID NO 1
1742-1747	*Bgl*II/*Bam*HI junction
1748-1763	*Per 5* exon1 nt 4410-4425 SEQ ID NO 1
1764-2396	*Per5* intron nt 4426-5058 SEQ ID NO 1
2397-2405	*Per5* exon2 nt 5059-5067 SEQ ID NO 1
2406-2411	*Nco*I site
2408-4215	β glucuronidase gene (GUS)
4217-4264	sequence from pB1221
4280-4652	*Per5* 3' UTR nt 6067-6439 SEQ ID NO 1
4653-4869	synthetic linker
4870-5121	CaMV DNA nt 7093-7344
5122-5129	linker
5130-5476	CaMV DNA nt 7093-7439
5477-5496	linker
5497-5606	synthetic MSV leader(MSV nt 167-186, 188-277)
5608-5613	*Bgl*II/*Bcl*I junction
5608-5698	*Adh1*.S nt 119-209
5699-5820	*Adh1*.S nt 555-672 plus 4 bases linker sequence
5821-5827	*Bam*HI/*Bgl*II junction
5828-5864	MSV nt 278-317
5863-5868	*Nco*I site
5865-6419	phosphinothricin acetyl transferase gene (Basta™ resistance selectable marker)
6420-6699	*nos* 3' UTR
6700-9335	pUC19 sequences

Because intron flanking sequences (exon DNA) have been shown to be important in the processing of the intron (Luehrsen and Walbot (1991)), 16 bases of flanking exon DNA were included the fusion within the *per5* untranslated leader.

Construction of PIGP/367. The promoter from the *per5* gene was amplified using the forward primer GGGGGATCCTCTAGACAATGATATACATAGATAAAAACC (SEQ ID NO 20) which introduces a *BamH*I (GGATCC) site 5' of the promoter to facilitate cloning. The reverse primer within the untranslated leader of the *per5* gene was GGGAGATCTCCTTCGCTGTACTATGTTATAAGAGAAGAG (SEQ ID NO 21) and introduced a *Bgl*II (AGATCT) restriction site 3'. Sequences homologous to the promoter are

underlined. The primers were synthesized on a 394 DNA/RNA Synthesizer (Applied Biosystems, Foster City, CA). Amplification reactions were completed with the Expand™ Long Template PCR System (Boehringer Mannheim, Indianapolis, IN). Plasmid perGen10.44, which contains 10.1 kb of the maize peroxidase gene and untranslated and non-transcribed sequences, was used as the template DNA. Amplifications were cycled with a 56°C annealing temperature. Amplification products were separated and visualized by 1.0% agarose gel electrophoresis. Resulting amplification products were excised from the agarose and the DNA was purified using Qiaex II (Qiagen, Hilden, Germany). The products were ligated into pCR2.1 using the Original TA Cloning Kit (Invitogen Corporation, San Diego, CA). Recombinant plasmids were selected on Luria agar (Gibco, Bethesda, MD) containing 75mg/liter ampicillin (Sigma, St Louis, MO) and 40 ml/plate of a 40mg/ml stock of X-gal (Boehringer Mannheim, Indianapolis, IN). Plasmid DNAs were purified using Wizard™ plus Miniprep DNA Purification System (Promega, Madison, WI). DNA was analyzed and subcloned with restriction endonucleases and T4 DNA ligase from Bethesda Research Laboratories (Bethesda, MD). The resultant *per5* promoter clone was named p121-20.

Intron 1 and 25 bases of flanking exon DNA from the *per5* gene was amplified using the forward primer GGGGGATCCTGACTGCTTTGTCAAGGTTCAATTCTGCTT (SEQ ID NO 22) which introduced a *Bam*HI (GGATCC) site 5' the exon/intron DNA, and the reverse primer, GGGCCATGGATCGCAGCCCTACACATGTAACAGTGTTGT (SEQ ID NO 23), which introduced an *Nco*I (CCATGG) site 3' to facilitate fusion at the ATG start codon of the GUS gene. Sequences homologous to the *per5* sequence are underlined. Amplification and cloning was completed as described above with the resultant intron clone named p122-2. The intron was then excised from p122-2 on the *Bam*HI/*Nco*I fragment and introduced 5' to the GUS gene/per 5 3' untranslated region in BSGUSper4. Ligations were transformed into DH5α (Laboratory, Bethesda, MD) and DNA was extracted as described above. Sequence across the junction was verified using Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer, Foster City, CA) and 373A DNA Sequencer (Applied Biosystems, Foster City, CA). Computer analysis of the sequences was facilitated by Sequencher™ 3.0 (Gene Codes Corporation, Ann Arbor, MI). The intermediate, p128-1, was then digested with *Bam*HI and ligated to the purified promoter *Bgl*II/*Bam*HI fragment from p121-20. To generate a final construct containing the selectable marker gene for

Basta™ resistance, the *per5* promoter/per5 intron/GUS gene/per5 3' UTR were excised from PIPG147-2 on a *PvuII/NotI* fragment and introduced into a *PmeI/NotI* site of pDAB367. pDAB367, which contains the gene for Basta™ resistance, is described in Example 27. The final construct was designated pPIGP/367.

Example 18

Transformation of Maize with pPIGP/367

A. Establishment of Type II Callus Targets. The materials and methods used were the same as in Example 10.

B. Helium Blasting and Selection. The materials and methods used were the same as in Example 10. Thirty three Basta™ resistant lines. designated pPIGP-01 thru pPIGP-33, were obtained.

C. Plant Regeneration. The materials and methods used were the same as in Example 8. Plantlets were regenerated from five of the PIGP/367 transgenic lines (PIGP/367-01, PIGP/367-06, PIGP/367-19, PIGP/367-32 and PIGP/367-33).

D. GUS histochemical staining. Tissue from plantlets of pPIGP-01 were histochemically evaluated as described in Example 10. The plantlets showed good GUS expression in the roots except for the root cap where no expression was observed. No expression was observed in the leaves of these young plants.

F. Protein Extraction and measurement of GUS. Leaf and root tissue was collected and analysis for GUS expression completed from four of the PIGP/367 transgenic lines (PIGP/367-06, PIGP/367-19, PIGP/367-32 and PIGP/367-33) which showed positive GUS histochemical expression. An untransformed plant at the same stage of development, CS405, served as a negative control. The 6th leaf and cleaned roots (roots were cleaned under cold running tap water and rinsed with distilled water) were collected from 4-5 R_0 plants plants within transgenic lines. The samples were either stored at - 70° C or powdered using liquid nitrogen. Fifty mL tubes, chilled on dry ice, were filled to 10 mL mark with powdered samples. Protein from each sample was extracted in duplicate. Four volumes/weight of extraction buffer (Extraction buffer is 1% polyvinylpolypyrrolidone (hydrated in the solution for at least one hour), 20% glycerol, 0.7 μL/mL β-mercaptoethanol, 50 mM NaPO; pH 7.0, 10 mM EDTA, 0.1% Triton X-100, 0.1% sarcosyl, 10 mM β-mercaptoethanol) was added to each sample. Samples were ground using Ultra-Turrax T 25 (IKA-Works INC, Staufen I. Br., W. Germany) and kept on ice. Samples were spun at 3000 rpm at 4° C for five minutes.

Ten µL/mL of protease inhibitor (50 µg/mL antipain, 50 ug/mL leupeptin, 0.1 mM chymostain, 5 µg/mL pepstatin, 0.24 µg/mL pefabloc (Boehringer Mannheim, Indianapolis, IN)) was added to withdrawn sample supernatant. The samples were then spun at 4° C for 10 minutes at 13,000 rpm. The supernatants were withdrawn and stored at - 70°C. Protein concentration was measured on a UV-Visible Spectrophotometer (Shimadzu, Kyoto, Japan). Five µL of sample was added to 2.5 mL of protein dye reagent (Sigma Diagnostics, St. Louis, Mo) and 100 µL of sterile water. A range of standards was made from protein standard solution (Sigma Diagnostics, St. Louis, Mo).

GUS activity was measured using a GUS-Light ™ Kit (Tropix Inc., Bedford, MA) in replicate samples of the duplicate extractions. Five µL samples of undiluted extract or of extract diluted so that the luminescence was within the range measured by the luminometer was added to 195 µL of the GUS-™ Diluent Solution. After 1 hr incubation, at 28° C in the dark, luminescence was measured using a Bio Orbit 1251 luminometer, equipped with a Bio Orbit 1291 injector, after injection of 300 µL of GUS-Light ™ Accelerator. Luminescence was integrated for 5 sec after a 5 sec delay. The standards used were extraction buffer, non-transformed tissue stock and GUS-Light ™ Gus Standard. The results are summarized in Table 17 and showed high levels of expression in the roots, but low to no significant expression in the leaves.

Table 17: Expression of GUS with PIGP/367 in Plants from Four Transformation Events

Line	Leaf (RLU/µg protein)	Root (RLU/µg protein)
PIGP/367-06	734	5735
PIGP/367-19	49	5745
PIGP/367-32	8	349
PIGP/367-33	72	1586
CS405	1	13

G. Summary of Expression Results. In the previous examples herein, no significant expression was observed in any maize tissue (although it was in rice) in the absence of an intron downstream from the *per5* promoter. When the *Adh1* intron was fused to the promoter (Examples 8, 10), expression in maize was observed. The *Adh1* intron I was not capable of restoring the root-preferential expression in maize that is characteristic of the native *per5* gene. Root-preferential expression was only achieved when the promoter was placed in combination with the *per5* intron. This is the first demonstration of an intron

directing tissue specific or tissue-preferential expression in transgenic plants. Xu *et al.* (1994) have reported preliminary studies on the promoter of another root-preferential gene, the triosephosphate isomerase gene from rice. They found that an intron is required for expression from this promoter in rice protoplasts, but the effects of the intron on gene expression in mature tissues has not been described.

The mechanism for enhancement by an intron is not well understood. The effect appears to be post-transcriptional (rather than promoter-like effects on the initiation of transcription) because the enhancements are only seen when the intron is present in the region of DNA that is transcribed (Callis, 1987). Introns could play a role in stabilizing the pre-mRNA in the nucleus, or in directing subsequent processing (Luehrsen and Walbot, 1991). The root-preferential expression of the *per5* promoter-intron combination could be explained by requiring an intron for processing, and a limited tissue distribution of other factor(s) necessary for correct processing.

Example 19

Plasmid p188-1

Plasmid p188-1 is a clone of the *per5* 3'UTR. The *per5* 3' UTR was amplified on Plasmid Xba4, which contains the 4.1 kb *Xba*I fragment from nt 2532 to 6438 of SEQ ID NO 1, using the forward primer, AAA GAG CTC TGA GGG CAC TGA AGT CGC TTG ATG TGC (SEQ ID NO 24), which introduced a SstI site on the 5' end, and the reverse primer, GGG GAA TTC TTG GAT ATA TGC CGT GAA CAA TTG TTA TGT TAC (SEQ ID NO 25), which introduced an *Eco*RI site on the 3' end of a 366 bp segment of *per5* 3' UTR (corresponding to nt 6066 to 6431 of SEQ ID NO 1). Sequences homologous to the promoter are underlined. The primers were synthesized on a 394 DNA/RNA Synthesizer (Applied Biosystems, Foster City, CA). Amplification reactions were completed with the Expand™ Long Template PCR System (Boehringer Mannheim, Indianapolis, IN). Plasmid Xba amplifications were cycled with a 56°C annealing temperature. Amplification products were separated and visualized by 1.0% agarose gel electrophoresis. Resulting amplification products were excised from the agarose and the DNA was purified using Qiaex II (Qiagen, Hilden, Germany). The products were ligated into pCR2.1 from the Original TA Cloning Kit (Invitrogen Corporation, San Diego, CA).

Recombinant plasmids were selected on Luria agar (Gibco, Bethesda, MD) containing 75mg/liter ampicillin (Sigma, St Louis, MO) and 40 ml/plate of a 40mg/ml stock of X-gal (Boehringer Mannheim, Indianapolis, IN). Plasmid DNAs were purified using

Wizard™ plus Miniprep DNA Purification System (Promega, Madison, WI). DNA was analyzed and subcloned with restriction endonucleases and T4 DNA ligase from Bethesda Research Laboratories (Bethesda, MD). The resultant *per5* 3'UTR clone was named p188-1.

Example 20
pTGP190-1

Plasmid pTGP190-1 is a 5887 bp plasmid comprising a gene cassette in which the following components are operably joined: the 35T promoter, the GUS gene, and the *per5* 3'UTR. The complete sequence of pTGP190-1 is given in SEQ ID NO 26. With reference to SEQ ID NO 26, important features of pTGP 190-1 include:

Table 18: Significant Features of pTGP 190-1

nt (SEQ ID NO 26)	Features
12-17	*Pst*I site
18-30	linker
31-282	CaMV MCASTRAS nt 7093-7344
283-290	linker
291-637	CaMV DNA MCASTRAS 7093-7439
638-657	linker
650-655	*Bam*HI site
651-1024	374 bp *Bam*HI/*Nco*I fragment containing MSV leader and *Adh1* intron
658-677	MSV nt 167-186
678-767	MSV nt 188-277
769-774	*Bgl*II/*Bcl*I junction
769-978	*Adh1*.S intron with deletion described in Example 24
979-988	linker
982-987	*Bam*HI/*Bgl*II junction
989-1028	MSV nt 278-317
1024-1029	*Nco*I site
1026-2834	β glucuronidase coding sequence (GUS)
2835-2890	sequence from pKA882
2890-2895	*Sst*I site
2896-3261	*Per5* 3'UTR nt 6066 to 6431 of SEQ ID NO 1
3262-3267	*Eco*RI site
3268-5897	pUC19 sequences

Construction of pTGP190-1. The *per5* 3' UTR was excised from p188-1 (Example 19) using the *Sst*I/*Eco*RI sites and purified from an agarose gel as described above. This fragment was ligated to the *Sst*I/*Eco*RI A fragment of pDAB305. (pDAB305 is described in detail in Example 24.) Plasmid pDAB305 is a 5800 bp plasmid that contains a heterologous promoter which is known as 35T. Construction of the 35T promoter is described in detail in Example 24. Basically this construct contains tandem copies of the Cauliflower Mosaic Virus 35S promoter (35S), a deleted version of the *Adh1* intron 1, and the untranslated

leader from the Maize Streak Mosaic Virus (MSV) Coat Protein fused to the β-glucuronidase gene, which is then followed by the *nos* 3'UTR.) The *SstI/Eco*RI A fragment of pDAB305 deletes the *nos* 3'UTR. Ligations were transformed into DH5α (Bethesda Research Laboratory, Bethesda, MD) and DNA was extracted as described above. Sequence across the promoter/GUS junction was verified using Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer, Foster City, CA) and 373A DNA Sequencer (Applied Biosystems, Foster City, CA). Computer analysis of the sequences was facilitated by Sequencher™ 3.0 (Gene Codes Corporation, Ann Arbor, MI). Plasmid pTGP190-1 is identical to pDAB305 except for the substitution of the *per5* 3'UTR for the *nos* 3'UTR following the GUS gene.

Example 21
UGP232-4

Plasmid UGP232-4 is similar to pTGP190-1, but contains the ubiquitin 1 (*ubi*) promoter and intron I from maize in place of the 35T promoter. The *ubi* promoter was excised on a *Hin*dIII/*Nco*I fragment from pDAB1538 (described in Example 29) and ligated to the *Hin*dIII/*Nco*I A fragment of pTGP190-1 to derive UGP232-4. The complete sequence for UGP232-4 is given in SEQ ID NO 27. With reference to SEQ ID NO 27, important features of UGP232-4 are given in Table 19.

Table 19: Significant Features of UGP232-4

nt (SEQ ID NO 27)	Features
1-5	*Hin*dIII site
1-14	pUC19 polylinker
15-993	ubiquitin promoter from maize
994-2007	ubiquitin intron
2008-2026	Synthetic polylinker from previous constructs (*Kpn*I, *Sma*I and *Sal*I)
2025-2030	*Nco*I site
2027-3835	β glucuronidase coding sequence (GUS)
3836-3890	sequence from pKA882
3891-3896	*Sst*I site
3897-4262	*Per5* 3' UTR nt 6066 to 6431 of SEQ ID NO 1
4263-4268	*Eco*RI site
4269-6898	pUC19 sequence

pUGN81-3 was used as the Ubiquitin/GUS/*nos* control plasmid.

Example 22
Quantitative Transient Assays of Maize Callus Bombarded with pTGP191-1 or UGP232-4

A. Preparation of DNA for transient testing. Each of the test constructs, in addition to pDAB305 (described in Example 24), was co-precipitated onto gold particles with pDeLux (described in Example 26) according to the following protocol. Equal molar amounts of the GUS constructs were used. A total of 140 µg of DNA, 70 µg of pDeLux plus 70 µg of test DNA and Bluescript® II SK⁻ DNA (when necessary), was diluted in sterile water to a volume of 300 µL. The DNA and water were added to 60 mg of surface-sterilized 1.0 µm spherical gold particles (Bio-Rad Laboratories, Hercules, CA). The mixture was vortexed briefly (approximately 15 seconds) before adding 74 µL of 2.5 M calcium chloride and 30 µL of 0.1 M spermidine (free base). After vortexing for 30 seconds, the DNA and gold were allowed to precipitate from solution. The supernatant was removed and 1 mL of ethanol was added. The DNA/gold mixture was diluted 1:8 before use for transformation.

B. Transient testing in maize callus. Regenerable (Type II) maize callus was pretreated on osmotic medium (N6 salts and vitamins (Chu (1978)), 1 mg/L 2,4-dichlorophenoxyacetic acid, 0.2 M sorbitol, 0.2 M mannitol, 7 g/L Gelrite, pH 5.8) for approximately 16 hours. Afterward, it was placed onto 60 x 20 mm plates of osmotic medium solidified with 2% agar for helium blasting. Cages of 104 µm mesh screen covered each "target" (500-600 mg of callus) to prevent splattering and loss of tissue. Targets were individually blasted with DNA/gold mixture using the helium blasting device described in Example 10. Under a vacuum of 650 mm Hg, at a shooting distance of 10 cm and pressure of 1500 psi, DNA/gold mixture was accelerated toward each target four times, delivering 20 µL per shot. The targets were rotated 180° after each blast. The tissue was also mixed halfway through the blasting procedure to expose unblasted callus. Upon completion of blasting, the targets were again placed onto the original osmotic medium for overnight incubation at 26°C in the dark.

Four Type II callus cell lines were selected for each experiment. Two targets from each line were used per treatment group. Also, two nontransformed controls (NTC) were included within each experiment, composed of tissue pooled from all four lines. These

controls were transferred to osmotic and blasting media according to the protocol above, but were not subjected to helium blasting.

C. GUS quantitative analysis. Approximately 20 hours after blasting, 200-400 mg of each target was transferred to a 1.5 mL sample tube (Kontes, Vineland, NJ). For extraction of proteins, callus was homogenized using a stainless steel Kontes Pellet Pestle powered by a .35 amp, 40 Watt motor (Model 102, Rae Corporation, McHenry, IL), at a setting of "90". Cell Culture Lysis Reagent from a Luciferase Assay kit (Promega, Madison, WI) served as the extraction buffer. Protease inhibitors, phenylmethylsulfonyl fluoride (PMSF) and leupeptin hemisulfate salt, were added to the lysis buffer at the concentrations of 1 mM and 50 μM, respectively. Before grinding, 0.5 μL of lysis buffer per mg tissue was added to the sample tube. The callus was homogenized in four 25-second intervals with a 10-second incubation on ice following each period of grinding. Afterward, 1.0 μL of lysis buffer per mg tissue was added to the sample which was maintained on ice until all sample grinding was completed. The samples were then centrifuged twice at 5°C for 7 minutes at full speed (Eppendorf Centrifuge Model 5415). After the first spin, the supernatant from each tube was removed and the pellet was discarded. Callus extracts (supernatants) were also collected after the second spin and maintained on ice for GUS and Luciferase (LUC) analyses.

From the LUC Assay kit, LUC Assay Buffer was prepared according to the manufacturer's instructions by reconstituting lyophilized luciferin substrate. This buffer was warmed to room temperature and loaded into the dispensing pump of an automatic luminescence photometer (Model 1251 Luminometer and Model 1291 Dispenser, Bio-Orbit, Finland). Each sample was tested in triplicate by adding 20 μL of extract to three polypropylene luminometer vials (Wallac, Gaithersburg, MD). Per vial, 100 μL of assay buffer was dispensed, and luminescence was detected over a 45-second integration period. "Blank reactions", including 20 μL of extraction buffer rather than callus extract, were also measured within each experiment to determine the extent of background readings of the luminometer.

For analysis of GUS activity, a GUS-Light™ assay kit (Tropix, Bedford, MA) was used. Again, each sample was tested in triplicate, using 20 μL of extract per luminometer vial. GUS-Light™ Reaction Buffer was prepared from the assay kit by diluting liquid

Glucuron™ substrate according to the manufacturer's instructions. This buffer was warmed to room temperature and added in 180 µL aliquots to each luminometer vial at 7-second intervals. After a one hour incubation at room temperature, 300 µL of GUS-Light™ Light Emission Accelerator Buffer was added and luminescence was detected over a 5-second integration period. "Blank reactions" were also included in the GUS assay, using 20 µL of extraction buffer rather than callus extract.

GUS and LUC results were reported in relative light units (RLU). Both "blank" and NTC readings were subtracted from sample RLU levels. For comparison of one construct to another, GUS readings were normalized to LUC data by calculating GUS/LUC ratios for each sample tested. The ratios for all samples within a treatment group were then averaged and the means were subjected to a T-test for determination of statistical significance. Within each experiment, results were reported as a percent of pDAB305 expression.

Transient bombardment of Type II callus for each of the constructs was completed as described above. By including pDAB305 as a standard in each experiment and reporting results as a percent of the standard, data from numerous experiments could be meaningfully compared. Table 20. lists results from three experiments testing the *nos* versus the *per5* 3'UTRs using two promoters. With either the 35T or *Ubi1* promoter, the *per5* 3'UTR resulted in higher transient GUS expression than the *nos* 3' end constructs. pUGN223-3 is a plasmid that contains a fusion of the maize ubiquitin promoter and ubiquitin intron 1 to the GUS gene similar to pUGP232-4. However, pUGN223-3 has the *nos 5* 3'UTR instead of the *per* 3'UTR. pUGN223--3 was used as a control to directly compare expression relative to the 3'UTRs of *per5* and *nos* in combination with the maize ubiquitin 1 (*Ubi1*) promoter and intron 1.

Table 20. Summary of transient GUS expression for all of the constructs tested.

Construct	GUS/LUC Ratio (% of pDAB305)
pDAB305 (35T/GUS/*nos*) (control)	*100
pTGP190-1 (35T/GUS/per5)	*114
pUGN223-3 (Ubi/GUS/*nos*) (control)	†137
pUGP232-4 (Ubi/GUS/per5)	†163

* not significantly different (p=0.05)
†significantly different (p=0.05)

Transient analysis indicated that the *per5* 3' UTR functioned as well as *nos* when the GUS gene was driven by the 35T promoter and 19% better than *nos* when driven by the maize Ubiquitin 1 promoter. The reason for this increased efficiency is not known, but it

could result from changes in the efficiency of processing or increased stability of the message.

Example 23

Comparison of GUS Expression in Transformed Rice for *Per5* 3' UTR and *nos* 3' UTR Constructs

This example measures quantitative GUS expression levels obtained when the 3' UTR is used as a polyadenylation regulatory sequence, UGP232-4, in transgenic rice plants. In this example the GUS gene is driven by the maize ubiquitin1 (Ubi1) promoter. Expression levels are compared with the nos 3' UTR sequence and the same promoter (Ubi1)/GUS fusion, pDAB1518 (described in Example 28).

A. Transgenic Production. As described in Example 9.

1. Plasmids. The plasmid UGP232-4, containing the GUS gene driven by the maize ubiquitin1 promoter and the Per5 3' UTR was described in Example 21. The plasmid pDAB354, which carries a gene for hygromycin resistance, was described in Example 25.

2. Rice Transformation. Production of transgenic rice plants was described in Example 9.

B. Expression Analysis. Analysis of GUS expression and Southern analysis techniques were described in Example 9. These results are summarized in Table 21 for 30 independent transgenic events recovered with UGP232-4 and 8 independent events from the control plasmid, pDAB1518 (described in Example 28).

Table 21: GUS Expression in Transformed Rice Plants For PER5 and NOS 3' UTR Constructs

Transgenic Event	GUS Activity (RLU / µg protein) Root	Leaf	Presence of Intact Construct
354/UGP-45	349,310	295,012	YES
354/UGP-36	326,896	172,316	YES
354/UGP-39	152,961	127,619	YES
354/UGP-40	126,027	106,275	YES
354/UGP-02	58,359	21,720	YES
354/UGP-03	54,509	20,758	YES
354/UGP-04	54,501	20,838	YES
354/UGP-10	53,222	26,514	YES
354/UGP-37	45,288	90,428	YES
354/UGP-34	43,226	7,180	NO*
354/UGP-48	37,284	28,029	YES
354/UGP-29	35,630	14,631	NO*
354/UGP-28	32,177	16,317	YES
354/UGP-19	29,646	13,143	NO*
354/UGP-31	29,520	19,774	YES
354/UGP-50	11,320	9,752	YES
354/UGP-44	9,301	9,556	NO*
354/UGP-35	7,113	2,062	YES

354/UGP-17	4,590	3,350	YES
354/UGP-27	3,367	975	YES
354/UGP-38	1,567	258	YES
354/UGP-22	1,202	1,229	YES
354/UGP-12	903	15	YES
354/UGP-42	670	780	NO*
354/UGP-11	378	96	YES
354/UGP-26	160	80	YES
354/UGP-25	152	340	YES
354/UGP-18	77	26	YES
354/UGP-06	69	95	YES
354/UGP-24	43	26	YES
1518-03	278,286	108,075	n.d.
1518-08	140,952	42,367	n.d.
1518-09	97,769	83,209	n.d.
1518-24	84,844	45,807	n.d.
1518-23	47,734	62,279	n.d.
1518-07	2,406	3,146	n.d.
1518-10	2,188	1,759	n.d.
1518-04	44	52	n.d.

* The expected 3.9 kb fragment was not obtained but instead a range of 2 to 4 other hybridization bands were noted.

n.d. = not determined

For both constructs there was a great deal of variability of GUS expression observed in both roots and leaves. Although a few events displayed higher GUS expression with the UGP construct, overall the expression levels using the *per5* 3' UTR were comparable to that of the *nos* 3' UTR. Southern analysis of plants from the 30 UGP232-4 events verified a corresponding 3.9 kb fragment to the GUS probe for the majority of events. Overall, the *per5* 3' UTR demonstrates the ability to augment expression as good, or better than the *nos* 3' UTR. The *per5* 3' UTR has also been used to express the GUS reporter gene in stably transformed maize (Examples 16). Therefore, this sequence has broad utility as a 3' UTR for expression of transgenic products in monocots, and probably in dicots.

Various combinations of the regulatory sequences from the *Per5* gene have proven to have utility in driving the expression of transgenic products in multiple crops. Table 22 summarizes the transient and stable expression patterns observed from each of the constructs tested in maize and the stable expression patterns observed in rice. These data demonstrate the ability of any of the *per5* promoter iterations to drive transgene expression. An unexpected finding was that introns significantly affect tissue specificity of transgene expression in stably transformed maize plants, but do not similarly affect expression in rice. In stably transformed maize plants the Adh1 intron supported expression in all tissues,

whereas the *per5* intron supported a tissue preferential pattern of expression. Finally, the *per5* 3' UTR was capable of supporting transgenic expression when used in combination with the *per5* promoter or other heterologous promoters in maize or rice.

Table 22. Summary of GUS expression patterns observed from various *per5* elements.

Promoter	Intron	3'UTR	Transient (root)	Stable Maize	Stable Rice
per5		nos	positive (low)	negative	n.d.
per5		per5	positive	negative	constitutive
per5	adh1	nos	positive	constitutive	constitutive
per5	per5	per5	n.d.	root specific	n.d.
35T	adh1	per5	positive	n.d.	n.d.
ubi	ubi	nos	positive (high)	n.d.	constitutive
ubi	ubi	per5	positivie (high)	n.d.	constitutive

n.d.= not determined

Example 24
pDAB 305

Plasmid pDAB305 is a 5800 bp plasmid that harbors a promoter containing tandem copy of the Cauliflower Mosaic Virus 35S enhancer (35S), a deleted version of the *Adh1* intron 1, and the untranslated leader from the Maize Streak Mosaic Virus Coat Protein fused to the β-glucuronidase gene, which is then followed by the *nos* 3'UTR.

A. Construction of a doubly-enhanced CaMV 35S Promoter.

This section describes molecular manipulations which result in a duplication of the expression-enhancer element of a plant promoter. This duplication has been shown (Kay et al (1987)) to result in increased expression in tobacco plants of marker genes whose expression is controlled by such a modified promoter. [Note: The sequences referred to in this discussion are derived from the Cabb S strain of Cauliflower Mosaic Virus (CaMV). They are available as the MCASTRAS sequence of GenBank, which is published. (Franck *et al.*, 1980). All of the DNA sequences are given in the conventional 5' to 3' direction. The starting material is plasmid pUC13/35S(-343) as described by Odell *et al.* (1985). This plasmid comprises, starting at the 3' end of the *Sma*I site of pUC13 (Messing(1983)) and reading on the strand contiguous to the noncoding strand of the lacZ gene of pUC13, nucleotides 6495 to 6972 of CaMV, followed by the linker sequence CATCGATG (which contains a *Cla*I recognition site), followed by CaMV nucleotides 7089 to 7443, followed by the linker sequence CAAGCTTG, the latter sequence comprising the recognition sequence for *Hin*dIII, which is then followed by the remainder of the pUC13 plasmid DNA.

1. pUC13/35S(-343) DNA was digested with *Cla*I and *Nco*I, the 3429 base pair (bp) large fragment was separated from the 66 bp small fragment by agarose gel electrophoresis, and then purified by standard methods.

2. pUC13/35S(-343) DNA was digested with *Cla*I, and the protruding ends were made flush by treatment with T4 DNA polymerase. The blunt-ended DNA was the ligated to synthetic oligonucleotide linkers having the sequence CCCATGGG, which includes an *Nco*I recognition site. The ligation reaction was transformed into competent *Escherichia coli* cells, and a transformant was identified that contained a plasmid (named pOO#1) that had an *Nco*I site positioned at the former *Cla*I site. DNA of pOO#1 was digested with *Nco*I and the compatible ends of the large fragment were religated, resulting in the deletion of 70 bp from pOO#1, to generate intermediate plasmid pOO#1 NcoΔ.

3. pOO#1 NcoΔDNA was digested with *Eco*RV, and the blunt ends were ligated to *Cla*I linkers having the sequence CATCGATG. An *E. coli* transformant harboring a plasmid having a new *Cla*I site at the position of the previous *Eco*RV site was identified, and the plasmid was named pOO#1 NcoΔ RV>Cla.

4. DNA of pOO#1 NcoΔ RV>Cla DNA was digested with *Cla*I and *Nco*I, and the small (268 bp) fragment was purified from an agarose gel. This fragment was then ligated to the 3429 bp *Cla*I/NcoI fragment of pUC13/35S(-343) prepared above in step 1, and an *E. coli* transformant that harbored a plasmid having *Cla*I/*Nco*I fragments 3429 and 268 bp was identified. This plasmid was named pUC13/35S En.

5. pUC13/35S En DNA was digested with *Nco*I, and the protruding ends were made blunt by treatment with T4 DNA polymerase. The treated DNA was then cut with *Sma*I, and was ligated to *Bgl*II linkers having the sequence CAGATCTG. An *E. coli* transformant that harbored a plasmid in which the 416 bp *Sma*I/*Nco*I fragment had been replaced with at least two copies of the *Bgl*II linkers was identified, and named p35S En^2. [NOTE: The tandomization of these *Bgl*II linkers generate, besides *Bgl*II recognition sites, also *Pst*I recognition sites, CTGCAG].

The DNA structure of p35s En^2 is as follows: Beginning with the nucleotide that follows the third C residue of the *Sma*I site on the strand contiguous to the noncoding strand of the *lacZ* gene of pUC13; the linker sequence CAGATCTGCAGATCTGCATGGGCGATG (SEQ ID NO 28), followed by CaMV nucleotides 7090 to 7344, followed by the *Cla*I linker sequence CATCGATG, followed by

CaMV nucleotides 7089 to 7443, followed by the *Hin*dIII linker sequence CAAGCTT, followed by the rest of pUC13 sequence. This structure has the feature that the enhancer sequences of the CaMV 35S promoter, which lie in the region upstream of the *Eco*RV site in the viral genome (nts 7090 to 7344), have been duplicated. This promoter construct incorporates the native 35S transcription start site, which lies 11 nucleotides upstream of the first A residue of the *HindIII* site.

B. Plasmids utilizing the 35S promoter and the *Agrobacterium nos* Poly A sequences.

The starting material for the first construct is plasmid pBI221, purchased from CLONTECH (Palo Alto, CA). This plasmid contains a slightly modified copy of the CaMV 35S promoter, as described in Bevan *et al.* (1985), Baulcombe *et al.* (1986), Jefferson *et al.*, (1986) and Jefferson (1987). Beginning at the 3' end of the Pst I site of pUC19 (Yanisch-Perron *et al.*(1985)) and reading on the same strand as that which encodes the *lacZ* gene of pUC19, the sequence is comprised of the linker nucleotides GTCCCC, followed by CaMV nucleotides 6605 to 7439 (as described in 24A), followed by the linker sequence GGGGACTCTAGAGGATCCCCGGGTGGTCAGTCCCTT (SEQ ID NO 29), wherein the underlined bases represent the *Bam*HI recognition sequence. These bases are then followed by 1809 bp comprising the coding sequence of the E. coli *uidA* gene, which encodes the β-glucuronidase (GUS) protein, and 55 bp of 3' flanking bases that are derived from the E. coli genome (Jefferson, 1986), followed by the *Sac*I linker sequence GAGCTC, which is then followed by the linker sequence GAATTTCCCC (SEQ ID NO 30). These bases are followed by the RNA transcription termination/polyadenylation signal sequences derived from the *Agrobacterium tumefaciens* nopaline synthase (*nos*) gene, and comprise the 256 bp *Sau*3A I fragment corresponding to nucleotides 1298 to 1554 of DePicker *et al.* (1982), followed by two C residues, the *Eco*RI recognition sequence GAATTC, and the rest of pUC19.

1. pBI221 DNA was digested with *Eco*RI and *Bam*HI, and the 3507 bp fragment was purified from an agarose gel. pRAJ275 (CLONTECH, Jefferson, 1987) DNA was digested with *Eco*RI and *Sal*I, and the 1862 bp fragment was purified from an agarose gel. These two fragments were mixed together, and complementary synthetic oligonucleotides having the sequence GATCCGGATCCG (SEQ ID NO 31) and TCGACGGATCCG (SEQ ID NO 32) were added. [These oligonucleotides when annealed have protruding single-stranded ends

compatible with the protruding ends generated by *Bam*HI and *Sal*I.] The fragments were ligated together, and an *E.coli* transformant harboring a plasmid having the appropriate DNA structure was identified by restriction enzyme analysis. DNA of this plasmid, named pKA881, was digested with *Bal*I and *Eco*RI, and the 4148 bp fragment was isolated from an agarose gel. DNA pBI221 was similarly digested, and the 1517 bp *Eco*RI/*Bal*I fragment was gel purified and ligated to the above pKA881 fragment, to generate plasmid pKA882.

2. pKA882 DNA was digested with *Sac*I, the protruding ends were made blunt by treatment with T4 DNA polymerase, and the fragment was ligated to synthetic *Bam*HI linkers having the sequence CGGATCCG. An *E.coli* transformant that harbored a plasmid having *Bam*HI fragments of 3784 and 1885 bp was identified and named pKA882B.

3. pKA882B DNA was digested with *Bam*HI, and the mixture of fragments was ligated. An *E.coli* transformant that harbored a plasmid that generated a single 3783 bp fragment upon digestion with *Bam*HI was identified and named p35S/*nos*. This plasmid has the essential DNA structure of pBI221, except that the coding sequences of the GUS gene have been deleted. Therefore, CaMV nucleotides 6605 to 7439 are followed by the linker sequence GGGGACTCTAGAGGATCCCGAATTTCCCC (SEQ ID NO 33), where the single underlined bases represent an *Xba*I site, and the double underlined bases represent a *Bam*HI site. The linker sequence is then followed by the *nos* Polyadenylation sequences and the rest of pBI221.

4. p35S/*nos* DNA was digested with *Eco*RV and *Pst*I, and the 3037 bp fragment was purified and ligated to the 534 bp fragment obtained from digestion of p35S En^2 DNA with *Eco*RV and *Pst*I. An *E. coli* transformant was identified that harbored a plasmid that generated fragments of 3031 and 534 bp upon digestion with *Eco*RV and *Pst*I, and the plasmid was named p35S En^2/*nos*. This plasmid contains the duplicated 35S promoter enhancer region described for p35S En^2 in Example 24A Step 5, the promoter sequences being separated from the *nos* polyadenylation sequences by linker sequences that include unique *Xba*I and *Bam*HI sites.

C. Construction of a synthetic untranslated leader.

This example describes the molecular manipulations used to construct a DNA fragment that includes sequences which comprise the 5' untranslated leader portion of the major rightward transcript of the Maize Streak Virus (MSV) genome. The MSV genomic sequence was published by Mullineaux *et al.*, (1984), and Howell (1984), and the transcript

was described by Fenoll *et al.* (1988). The entire sequence, comprising 154 bp, was constructed in three stages (A, B, and C) by assembling blocks of synthetic oligonucleotides.

1. The A Block: Complementary oligonucleotides having the sequence GATCCAGCTGAAGGCTCGACAAGGCAGATCCACGGAGGAGCTGATATTTGGTGG ACA (SEQ ID NO 34) and AGCTTGTCCACCAAATATCAGCTCCTCCGTGGATCTGCCTTGTCCAGCCTTCAGC TG (SEQ ID NO 35) were synthesized and purified by standard procedures. Annealing of these nucleotides into double-stranded structures leaves 4-base single stranded protruding ends [hereinafter referred to as "sticky ends"] that are compatible with those generated by *Bam*HI on one end of the molecule (GATC), and with *Hin*dIII-generated single stranded ends on the other end of the molecule (AGCT). Such annealed molecules were ligated into plasmid Bluescript ® II SK$^-$ that had been digested with *Bam*HI and *Hin*dIII. The sequence of these oligonucleotides is such that, when ligated onto the respective *Bam*HI and *Hin*dIII sticky ends, the sequences of the respective recognition sites are maintained. An *E. coli* transformant harboring a plasmid containing the oligonucleotide sequence was identified by restriction enzyme analysis, and the plasmid was named pMSV A.

2. The B Block: Complementary oligonucleotides having the sequences AGCTGTGGATAGGAGCAACCCTATCCCTAATATACC AGCACCACCAAGTCAGGGCAATCCCGGG (SEQ ID NO 36) and TCGACCCGGGATTGCCCTGACTTGGTGGTGCTGGTATATTAGGGATAGGGTTGCT CCTATCCAC (SEQ ID NO 37) were synthesized and purified by standard procedures. The underlined bases represent the recognition sequence for restriction enzymes *Sma*I and *Xma*I. Annealing of these nucleotides into double-stranded structures leaves 4-base sticky ends that are compatible with those generated by *Hin*dIII on one end of the molecule (AGCT), and with *Sal*I-generated sticky ends on the other end of the molecule (TCGA). The sequence of these oligonucleotides is such that, when ligated onto the *Hin*dIII sticky ends, the recognition sequence for *Hin*dIII is destroyed.

DNA of pMSV A was digested with *Hin*dIII and *Sal*I, and was ligated to the above annealed oligonucleotides. An *E. coli* transformant harboring a plasmid containing the new oligonucleotides was identified by restriction enzyme site mapping, and was named pMSV AB.

3. The C Block: Complementary oligonucleotides having the sequences CCGGGCCATTTGTTCCAGGCACGGGATAAGCATTCAGCCATGGGATATCAAGCT TGGATCCC (SEQ ID NO 38) and TCGAGGGATCCAAGCTTGATATCCCATGGCTGAATGCTTATCCCGTGCCTGGAAC AAATGGC (SEQ ID NO 39) were synthesized and purified by standard procedures. The oligonucleotides incorporate bases that comprise recognition sites (underlined) for *Nco*I (CCATGG), *Eco*RV (GATATC), *Hin*dIII (AAGCTT), and *Bam*HI (GGATCC). Annealing of these nucleotides into double-stranded structures leaves 4-base sticky ends that are compatible with those generated by *Xma*I on one end of the molecule (CCGG), and with *Xho*I-generated sticky ends on the other end of the molecule (TCGA). Such annealed molecules were ligated into pMSV AB DNA that had been digested with *Xma*I and *Xho*I. An *E.coli* transformant harboring a plasmid containing the oligonucleotide sequence was identified by restriction enzyme analysis, and DNA structure was verified by sequence analysis. The plasmid was named pMSV CPL; it contains the A, B and C blocks of nucleotides in sequential order ABC. Together, these comprise the 5′ untranslated leader sequence ("L") of the MSV coat protein ("CP") gene. These correspond to nucleotides 167 to 186, and 188 to 317 of the MSV sequence of Mullineaux *et al.*, (1984), and are flanked on the 5′ end of the *Bam*HI linker sequence GGATCCAG, and on the 3′ end by the linker sequence GATATCAAGCTTGGATCCC (SEQ ID NO 40). [Note: An A residue corresponding to base 187 of the wild type MSV sequence was inadvertently deleted during cloning.]

4. *BglII* Site Insertion: pMSV CPL DNA was digested at the *Sma*I site corresponding to base 277 of the MSV genomic sequence, and the DNA was ligated to *Bgl*II linkers having the sequence CAGATCTG. An *E.coli* transformant harboring a plasmid having a unique *Bgl*II site at the position of the former *Sma* I site was identified and verified by DNA sequence analysis, and the plasmid was named pCPL-Bgl.

D. Construction of a deleted version of the maize alcohol dehydrogenase 1 (*Adh1*) intron 1

The starting material is plasmid pVW119, which was obtained from V. Walbot, Stanford University, Stanford, CA. This plasmid contains the DNA sequence of the maize *Adh1*.S gene, including intron 1, from nucleotides 119 to 672 [numbering of Dennis *et al.* (1984)], and was described in Callis *et al.* (1987). In pVW119, the sequence following base

672 of Dennis *et al.* (1984) is GACGGATCC, where the underlined bases represent a *Bam*HI recognition site. The entire intron 1 sequence, with 14 bases of exon 1, and 9 bases of exon 2, can be obtained from this plasmid on a 556 bp fragment following digestion with *Bcl*I and *Bam*HI.

1. Plasmid pSG3525a(Pst) DNA was digested with *Bam*HI and *Bcl*I , and the 3430 bp fragment was purified from an agarose gel. [NOTE: The structure of plasmid pSG3525a(Pst) is not directly relevant to the end result of this construction series. It was constructed during an unrelated series, and was chosen because it contained restriction recognition sites for both *Bcl*I and *Bam*HI, and lacks *Hin*dIII and *Stu*I sites. Those skilled in the art will realize that other plasmids can be substituted at this step with equivalent results.] DNA of plasmid pVW119 was digested with *Bam*HI and *Bcl*I , and the gel purified fragment of 546 bp was ligated to the 3430 bp fragment. An *E.coli* transformant was identified that harbored a plasmid that generated fragments of 3430 and 546 upon digestion with *Bam*HI and *Bcl*I . This plasmid was named pSG AdhA1.

2. DNA of pSG AdhA1 was digested with *Hin*dIII, [which cuts between bases 209 and 210 of the Dennis *et al.*, (1984) sequence, bottom strand], and with *Stu*I, which cuts between bases 554 and 555. The ends were made flush by T4 DNA polymerase treatment, and then ligated. An *E.coli* transformant that harbored a plasmid lacking *Hin*dIII and *Stu*I sites was identified, and the DNA structure was verified by sequence analysis. The plasmid was named pSG AdhA1Δ. In this construct, 344 bp of DNA have been deleted from the interior of the intron 1. The loss of these bases does not affect splicing of this intron. The functional intron sequences are obtained on a 213 bp fragment following digestion with *Bcl*I and *Bam*HI.

3. DNA of plasmid pCPL-Bgl (Example 24C Step 4), was digested with *Bgl*II, and the linearized DNA was ligated to the 213 bp *Bcl*I /*Bam*HI fragment containing the deleted version of the *Adh1*.S intron sequences from pSG AdhA1Δ. [Note: The sticky ends generated by digestion of DNA with *Bgl*II, *Bcl*I , and *Bam*HI are compatible, but ligation of the *Bam*HI or *Bcl*I sticky ends onto ones generated by *Bgl*II creates a sequence not cleaved by any of these three enzymes.] An *E.coli* transformant was identified by restriction enzyme site mapping that harbored a plasmid that contained the intron sequences ligated into the *Bgl*II site, in the orientation such that the *Bgl*II/*Bcl*I juncture was nearest the 5′ end of the

MSV CPL leader sequence, and the *BglII/ Bam*HI juncture was nearest the 3' end of the CPL. This orientation was confirmed by DNA sequence analysis. The plasmid was named pCPL A1113. The MSV leader/intron sequences can be obtained from this plasmid by digestion with *Bam*HI and *Nco*I, and purification of the 373 bp fragment.

E. Construction of plant expression vectors based on the enhanced 35S promoter, the MSV CPL, and the deleted version of the *Adh1* intron 1

1. DNA of plasmid p35S En^2/*nos* was digested with *Bam*HI, and the 3562 bp linear fragment was ligated to a 171 bp fragment prepared from pMSV CPL DNA digested with *Bam*HI. This fragment contains the entire MSV CPL sequence described in Example 7C. An *E.coli* transformant was identified by restriction enzyme site mapping that harbored a plasmid that contained these sequences in an orientation such that the *Nco*I site was positioned near the *nos* Poly A sequences. This plasmid was named p35S En^2 CPL/*nos*. It contains the enhanced version of the 35S promoter directly contiguous to the MSV leader sequences, such that the derived transcript will include the MSV sequences in its 5' untranslated portion.

2. DNA of plasmid pKA882 (see Example 24B Step 1) was digested with *Hin*dIII and *Nco*I, and the large 4778 bp fragment was ligated to an 802 bp *Hin*dIII/*Nco*I fragment containing the enhanced 35S promoter sequences and MSV leader sequences from p35S En^2 CPL/*nos*. An *E.coli* transformant harboring a plasmid that contained fragments of 4778 and 802 bp following digestion with *Hin*dIII and *Nco*I was identified, and named pDAB310. In this plasmid, the enhanced version of the 35S promoter is used to control expression of the GUS gene. The 5' untranslated leader portion of the transcript contains the leader sequence of the MSV coat protein gene.

3. DNA of plasmid pDAB310 was digested with *Nco*I and Sac I. The large 3717 bp fragment was purified from an agarose gel and ligated to complementary synthetic oligonucleotides having the sequences CGGTACCTCGAGTTAAC (SEQ ID NO 41) and CATGGTTAACTCGAGGTACCGAGCT (SEQ ID NO 42). These oligonucleotides, when annealed into double stranded structures, generate molecules having sticky ends compatible with those left by *Sac*I, on one end of the molecule, and with *Nco*I on the other end of the molecule. In addition to restoring the sequences of the recognition sites for these two enzymes, new sites are formed for the enzymes *Kpn*I (GGTACC), *Xho*I (CTCGAG), and *Hpa*I (GTTAAC). An *E. coli* transformant was identified that harbored a plasmid that

contained sites for these enzymes, and the DNA structure was verified by sequence analysis. This plasmid was named pDAB1148.

4. DNA of plasmid pDAB1148 was digested with *Bam*HI and *NcoI*, the large 3577 bp fragment was purified from an agarose gel and ligated to a 373 bp fragment purified from pCPL A111_ (Example 24D Step 3) following digestion with *Bam*HI and *Nco*I. An *E.coli* transformant was identified that harbored a plasmid with *Bam*HI and *Nco*I, and the plasmid was named pDAB303. This plasmid has the following DNA structure: beginning with the base after the final G residue of the *Pst*I site of pUC19 (base 435), and reading on the strand contiguous to the coding strand of the *lacZ* gene, the linker sequence ATCTGCATGGGTG (SEQ ID NO 43), nucleotides 7093 to 7344 of CaMV DNA, the linker sequence CATCGATG, nucleotides 7093 to 7439 of CaMV, the linker sequence GGGGACTCTAGAGGATCCAG (SEQ ID NO 44), nucleotides 167 to 186 of MSV, nucleotides 188 to 277 of MSV, a C residue followed by nucleotides 119 to 209 of *Adh1*.S, nucleotides 555 to 672 of maize *Adh1*.S, the linker sequence GACGGATCTG, nucleotides 278 to 317 of MSV, the polylinker sequence GTTAACTCGAGGTACCGAGCTCGAATTTCCCC (SEQ ID NO 45) containing recognition sites for *Hpa*I, *Xho*I, *Kpn*I, and *Sac*I, nucleotides 1298 to 1554 of *nos*, and a G residue followed by the rest of the pUC19 sequence (including the *Eco*RI site). It is noteworthy that the junction between nucleotide 317 of MSV and the long polylinker sequence creates an *NcoI* recognition site.

5. DNA of plasmid pDAB303 was digested with *Nco*I and *Sac*I, and the 3939 bp fragment was ligated to the 1866 bp fragment containing the GUS coding region prepared from similarly digested DNA of pKA882. The appropriate plasmid was identified by restriction enzyme site mapping, and was named pDAB305. This plasmid has the enhanced promoter, MSV leader and *Adh1* intron arrangement of pDAB303, positioned to control expression of the GUS gene.

Example 25

Plasmid pDAB354

All procedures were by standard methods as taken from Maniatis *et al.*, (1982).

Step 1: Plasmid pIC19R (Marsh *et al.*, (1984) was digested to completion with restriction enzyme *Sac*I, the enzyme was inactivated by heat treatment, and the plasmid DNA was ligated on ice overnight with an 80-fold excess of nonphosphorylated

oligonucleotide linker having the sequence 5' GAGTTCAGGCTTTTTCATAGCT 3' (SEQ ID NO 46), where AGCT is complementary to the overhanging ends generated by *Sac*I digestion. The linker-tailed DNA was then cut to completion with enzyme *Hin*dIII, the enzyme was inactivated, and the DNA precipitated with ethanol.

Step 2: Plasmid pLG62 contains a 3.2 Kb *Sal*I fragment that includes the hygromycin B phosphotransferase (resistance) gene as set forth in Gritz and Davies (1983). One microgram of these fragments was isolated from an agarose gel and digested to completion with restriction enzyme Hph I to generate fragments of 1257 bp. The enzyme was inactivated, and the 3' ends of the DNA fragments were resected by treatment with T4 DNA polymerase at 37° for 30 min in the absence of added deoxynucleotide triphosphates.

Step 3: Following inactivation of the polymerase and ethanol precipitation of the DNA, the fragments prepared in Step 2 were mixed in Nick Translation Salts (Maniatis *et al.*, 1982) with the linker-tailed vector prepared in Step 1, heated 5 min at 65°, and slowly cooled to 37°. The non-annealed ends were made blunt and single-stranded regions filled in by treatment with the Klenow fragment of *Escherichia coli* DNA polymerase by incubation at 37° for 45 min, and then the mixture was ligated overnight at 15°. Following transformation into *E. coli* MC1061 cells and plating on LB agar with 50 μg each of ampicillin and hygromycin B, an isolate was identified that contained a plasmid which generated appropriately-sized fragments when digested with *Eco*RI, *Pst*I, or *Hin*cII. DNA sequence determination of a portion of this plasmid (pHYG1) revealed the sequence 5' AGATCTCGTGAGATAATGAAAAAG 3', (SEQ ID NO 47) where the underlined ATG represents the start codon of the hygromycin B resistance gene, and AGATCT is the *BglII* recognition sequence. In pHYG1, downstream of the hygromycin B resistance coding region, are about 100 bases of undetermined sequence that were deleted in the next step.

Step 4: DNA of plasmid pHYG1 was digested to completion with restriction enzyme *Bam*HI, and the linear fragment thus produced was partially digested with *Sca*I. Fragments of 3644 bp were isolated from an agarose gel and ligated to phosphorylated, annealed complementary oligonucleotides having the sequences:

5'
ACTCGCCGATAGTGGAAACCGACGCCCCAGCACTCGTCCGAGGGCAAAGGAAT
AGTAAGAGCTCGG 3' (SEQ ID NO 48), and

5' GATCCCGAGCTCTTACTATTCCTTTGCC
CTCGGACGAGTGCTGGGGCGTCGGTTTCCACTATCGGCGAGT 3' (SEQ ID NO 49).
When annealed, these oligonucleotides have a protruding 4-base overhang on one end that is complementary to that generated by *Bam*HI. Following transformation of the ligation mixture into *E. coli* DH5α cells and selection on LB media containing 50 μg/ml of ampicillin, a transformant was identified that contained a plasmid which generated expected fragments when digested with *Bam*HI, *Bgl*II, *Eco*RI, or *Sac*I. This plasmid was named pHYG1 3'Δ. The sequence of this plasmid downstream from the stop codon of the hygromycin B resistance coding region (underlined TAG in above sequence; Gritz and Davies, 1983) encodes the recognition sequence for *Sac*I.

Step 5. DNA of plasmid pDAB309 was digested to completion with restriction enzyme *Bsm*I, and the ends were made blunt by treatment with T4 DNA polymerase. Plasmid pDAB309 has the same basic structure as pDAB305 described elsewhere herein, except that a kanamycin resistance (NPTII) coding region is substituted for the GUS coding region present in pDAB305. This DNA was then ligated to phosphorylated, annealed oligonucleotide *Bgl*II linkers having the sequence 5' CAGATCTG 3'. A transformed colony of DH5α cells harboring a plasmid that generated appropriately-sized fragments following *Bgl*II digestion was identified. This plasmid was named pDAB309(Bg). DNA of plasmid pDAB309(Bg) was cut to completion with *Sac*I, and the linearized fragments were partially digested with *Bgl*II. Fragments of 3938 bp (having ends generated by *Bgl*II and *Sac*I) were isolated from an agarose gel.

Step 6. DNA of plasmid pHYG1 3'Δ was digested to completion with *Bgl*II and *Sac*I. The 1043 bp fragments were isolated from an agarose gel and ligated to the 3938 bp *Bgl*II/*Sac*I fragments of pDAB309(Bg) prepared above. After transformation into *E. coli* DH5α cells and selection on ampicillin, a transformant was identified that harbored a plasmid which generated the appropriately-sized restriction fragments with *Bgl*II plus *Sac*I, *Pst*I, or *Eco*RI. This plasmid was named pDAB354. Expression of the hygromycin B resistance coding region is placed under the control of essentially the same elements as the GUS coding region in pDAB305.

Example 26
Plasmid pDeLux

Production of the GUS protein from genes controlled by different promoter versions was often compared relative to an internal control gene that produced firefly luciferase. DeWet et al (1987). A plasmid (pT3/T7-1 LUC) containing the luciferase (LUC) coding region was purchased from CLONTECH (Palo Alto, CA), and the coding region was modified at its 5' and 3' ends by standard methods. Briefly, the sequences surrounding the translational start (ATG) codon were modified to include an *Nco*I site (CCATGG) and an alanine codon (GCA) at the second position. At the 3' end, an *Ssp* I recognition site positioned 42 bp downstream of the Stop codon of the luciferase coding region was made blunt ended with T4 DNA polymerase, and ligated to synthetic oligonucleotide linkers encoding the *Bgl*II recognition sequence. These modifications permit the isolation of the intact luciferase coding region on a 1702 bp fragment following digestion by *Nco*I and *Bgl*II. This fragment was used to replace the GUS gene of plasmid pDAB305 (see Example 24E, step 5), such that the luciferase coding region was expressed from the enhanced 35S promoter, resulting in plasmid pDeLux. The 5' untranslated leader of the primary transcript includes the modified MSV leader/Adh intron sequence.

Example 27
Plasmid pDAB367

Plasmid pDAB367 has the following DNA structure: beginning with the base after the final C residue of the *Sph*I site of pUC 19 (base 441), and reading on the strand contiguous to the LacZ gene coding strand, the linker sequence CTGCAGGCCGGCCTTAATTAAGCGGCCGCGTTTAAACGCCCGGGCATTTAAATGGC GCGCCGCGATCGCTTGCAGATCTGCATGGGTG (SEQ ID NO 50), nucleotides 7093 to 7344 of CaMV DNA (Frank *et al.* (1980)), the linker sequence CATCGATG, nucleotides 167 to 186 of MSV (Mullineaux *et al.* (1984)), nucleotides 188 to 277 of MSV (Mullineaux *et al.* (1984)), a C residue followed by nucleotides 119 to 209 of maize Adh 1S containing parts of exon 1 and intron 1 (Denis *et al.* (1984)), nucleotides 555 to 672 containing parts of Adh 1S intron 1 and exon 2 (Denis *et al.* (1984)), the linker sequence GACGGATCTG (SEQ ID NO 51), and nucleotides 278 to 317 of MSV. This is followed by a modified BAR coding region from pIJ4104 (White *et al.* (1990)) having the AGC serine codon in the second position replaced by a GCC alanine codon, and nucleotide 546 of the coding region changed from G to A to eliminate a *Bgl*II site. Next the linker sequence TGAGATCTGAGCTCGAATTTCCCC

(SEQ ID NO 52) , nucleotides 1298 to 1554 of *nos* (DePicker *et al.* (1982)), and a G residue followed by the rest of the pUC19 sequence (including the *Eco*RI site.).

Example 28

Plasmid pDAB1518

pDAB1518 has the following DNA structure: the sequence CCGCGG, bases -899 to +1093 of the maize ubiquitin 1 (Ubi1) promoter and Ubi1 intron 1 described by Christensen *et al.* (1992), a polylinker consisting of the sequence GGTACCCCCGGGGTCGACCATGG (SEQ ID NO: 53) (containing restriction sites for *Kpn*I, *Sma*I, *Sal*I, and *Nco*I, with the *Nco*I site containing the translational fusion ATG), bases 306-2153 of the β-glucuronidase gene from pRAJ220 described by Jefferson *et al.* (1986), the sequence GGGAATTGGAGCTCGAATTTCCCC (SEQ ID NO: 54), bases 1298 to 1554 of *nos* (Depicker *et al.* (1982)), and the sequence GGGAAATTAAGCTT (SEQ ID NO: 55), followed by pUC18 (Yanisch-Perron *et al.*, 1985) sequence from base 398 to base 399 (reading on the strand opposite to the strand contiguous to the LacZ gene coding strand).

Example 29

Plasmid pDAB1538

pDAB1538 has the following DNA structure: the sequence AGCGGCCGCATTCCCGG GAAGCTTGCATGCCTGCAGAGATCCGGTACCCGGGGATCCTCTAGAGTCGAC (SEQ ID NO: 56), bases -899 to +1093 of the maize ubiquitin 1 (Ubi1) promoter and Ubi1 intron 1 described by Christensen *et al.* (1992), a polylinker consisting of the sequence GGTACCCCCGGGGTCGACCATGGTTAACTCGAGGTACCGAGCTCGAATTTCCCC (SEQ ID NO: 57), bases 1298 to 1554 of *nos* (Depicker *et al.* (1982)), and the sequence GGGAATTGGTTTAAACGCGGCCGCTT (SEQ ID NO:58), followed by pUC19 (Yanisch-Perron *et al.*, 1985) sequence starting at base 400 and ending at base 448 (reading on the strand opposite to the strand contiguous to the LacZ gene coding strand). The *Nco*I site in the Ubi1 sequence beginning at base 143 was replaced by the sequence CCATGCATGG (SEQ ID NO:59).

REFERENCES

Anderson (1984), Science, 226:401.

Armstrong *et al.* (1991), Maize Genet. Coop. New Lett. 65:92.

Austin, G. D. (1994), U. S. Patent no. 5,362,865.

Ausubel *et al.* (1987) Current Protocols in Molecular Biology, John Wiley and Sons, New York, NY.

Baulcombe *et al.*, (1986). Nature 321:446-449.

Beaucage *et al.* (1981), Tetrahedron Letters, 22:1859-1962).

Benfey P. N., L. Ren and N.-H. Chua. (1989), EMBO Journal 8:2195-2202.

Benfey, P. N., and Nam-Hai Chua. (1990), Science 250:959-966.

Bevan *et al.* (1985), EMBO J. 4:1921-1926.

Bevan *et al.* (1986) Nucleic Acids Res. 14 (11), 4675-4638.

Bohimann and Apel (1991), Annu. Rev. Plant Physiol Plant Mol. Biol., 42:227-240.

Bradford (1976) Anal. Biochem. 72: 248-254.

Brewbaker *et al.* (1985), Journal of Heredity, 76:159-167.

Buffard *et al.* (1990), Proc. Natl. Acad. Sci., 87:8874-8878.

Callis J., M. Fromm, and V. Walbot. (1987), Gene Dev. 1:1183 -1200.

Cammue *et al.* (1992), J. Biol. Chem, 267:2228-2233.

Caruthers (1983) in: Methodology of DNA and RNA, (ed.) Weissman.

Christensen, *et al.* (1992) Plant Mol. Biol. 18: 675-689.

Chu (1978), Proc. Symp. Plant Tissue Culture, Peking Press, p43-56.

Conkling *et al.* (1990), Plant Physiol., 93(3), 1203-1211.

Crossway, *et al.* (1986), Mol. Gen. Genet. 202:179-185.

Croy, *et al.*, WO 9113992

Datla, R.S.S. *et al.* (1993), Plant Science 94:139-149.

De Framond, EPO Application Number 452 269

Deikman *et al.* (1988), Embo J. 7 (11) 3315.

Dennis *et al.* (1984), Nucl. Acids Res. 12:3983-4000.

DePicker *et al.* (1982), J. Molec. Appl. Genet. 1: 561-573.

DeWet *et al.* (1987), Molec. Cell Biol. 7:725-737.

Dityatkin, *et al.* (1972), Biochimica et Biophysica Acta, 281:319-323.

EPO 0 405 696.

Erlich (ed.)(1989)).PCR Technology: Principles and Applications for DNA Amplification.

Fenoll *et al.* (1988), EMBO J. 7:1589-1596.

Fraley, *et al.* (1986), CRC Crit. Rev. Plant Sci., 4:1-46.

Frank *et al.* (1980) Cell 21:285-294.

Fu *et al.* (1995), The Plant Cell, 7:1387-1394.

Fujiyama *et al.* (1988), Eur. J. Biochem., 173:681-687.

Gallie *et al.* (1989), The Plant Cell, 1:301-311.

Gamborg *et al.* (1968), Exp. Cell Res. 50: 151-158.

Gaspar *et al.* (1982), Peroxidases: A Survey of Their Biochemical and Physiological Roles in Higher Plant (Univ. of Geneva Press, Geneva).

Gritz *et al.* (1983), Gene 25:179-188.

Grunstein, M. (1992), Scientific American, October 68-74.

Hertig *et al.* (1991), Plant Mol. Biol., 16:171-174.

Hiatt, *et al.* (1989), Nature, 342:76-78.

Higuchi *et al.* (1988), Nucl. Acids Res., 16:7351.

Higuchi *et al.* (1988), Nucl. Acids Res., 16:7351-7367.

Ho *et al.* (1989), Gene, 77:51-59.

Hofte and Whitely (1989), Microbiol. Rev., 53:242-255.

Horton *et al.* (1989), Gene, 77:61.

Howell (1984). Nucl. Acids Res. 12:7359-7375.

Hultmark *et al.* (1982), EUR. J. Biochem., 127:207-217.

Hultmark *et al.* (1983), EMBO J., 2:571-576.

Jackson and Standart (1990), Cell 62:15-24.

Jefferson (1987) Plant Molec. Biol. Reporter 5:387.

Jefferson *et al.* (1986), Proc. Natl. Acad. Sci. 83:8447-8451.

Jefferson *et al.* (1987), EMBO J. 6: 3901.

Kaiser *et al.* (1987), Ann. RevBiophys. Biophys. Chem., 16:561-581).

Kat *et al.* (1987), Science 236:1299.

Katsu *et al.* (1988), Biochim. Biophys. Acta, 939:57-63.

Kay *et al.* (1987), Science 236 1299-1302.

Knott *et al.* (1985), Science, 230:37.

Knowles *et al.* (1987), Biochim. Biophys. Acta 924:509-518.

Kozak (1986), Cell 44:283-292.

Kriz, A. L. *et al.* (1987), Molecular and General Genetics 207: 90-98.

Lagrimini *et al.* (1987), Plant Physiol., 84:438-442.

Lagrimini *et al.* (1987), Proc. Natl. Acad. Sci., 84:7542-7546.

MD.

Lee, K. and A. H. C. Huang. (1994), Plant Molecular Biology 26:1981-1987.

Lewah *et al.* (1991), J. Biol. Chem., 266:1564-1573.

Li *et al.* (1993), Plant Cell Rep. 12: 250-255.

Linthorst (1991), Critical Rev. Plant Sci., 10:123-150.

Lis *et al.* (1986), Ann. Rev. Biochem., 55:35-68.

Luehrsen, K. R. and V. Walbot. (1994), Plant Cell Reports 13:454-458.

Mandel and Higa (1972), J. Mol. Biol., 53:159.

Maniatis *et al.*, eds. (1982) Molecular Cloning, First Edition, Cold Spring Harbor Press.

Marsh *et al.* (1984), Gene 32:481.

Matzke *et al.* (1993), Ann. Rev. Plant Physiol. Plant Mol. Biol. 44: 53-76.

Mazza and Welinder (1980), Eur. J. Biochem. 108:481-489.

McElroy, D. and R. S. Brettell. (1994), Trends Biotechnology 12:62-68.

Messing *et al.* (1983) in:Genetic Engineering of Plants, (Kosuga *et al.* eds.), Plenum Press, pp. 211-227).

Miller, *et al.* (1988), Proc. Natl. Acad. Sci. USA, 85:856-860;

Morgens *et al.* (1990), Plant Mol. Biol., 14:715.

Mullineaux *et al.* (1984), EMBO J. 3:3063.

Mullis *et al.* (1987), Meth. Enz., 155:335.

Murashige and Skoog (1962), Physiol. Plant,15: 473.

Nakagawa *et al.*, (1985) J. Am. Chem. Soc., 107:7087;

Nakamura *et al.* (1988), Plant Physiol., 88:845.

Odell *et al.* (1985), Nature 313: 810-812.

Oiki *et al.* (1988), PNAS USA, 85:2393-2397.

Pain (1986), Biochem. J., 235:625-637.

Pear *et al.* (1989), Plant Mol. Biol. 13: 639.

Powell, et al (1988), Appl. Environ. Microbiol., 54:655-660.

Raju, S. S. D. et al (1993), Plant Science 94: 139-149.

Rothnie *et al.* (1994), EMBO Journal, 13:2200-2210.

Ryan (1990), Annu Rev. Phytopathol., 28:425.

Saghai-Maroof *et al.* (1984), Proc. Natl. Acad. Sci. USA 81:8014.

Samac *et al.* (1990), Plant Physiol. 93: 907-914

Sambrook *et al.* (1989), Molecular Cloning: A Laboratory Manual, 2nd edition, Cold Spring Harbor Press, Cold Spring Harbor, NY.

Shigekawa and Dower (1988), Biotechniques, 6:742.

Southern, E. (1975), J. Mol. Biol. 98:503.

Southern, E. (1980), Methods Enzymol. 69:152.

Sullivan, M. L and P. Green (1993), Plant Molecular Biology 23: 1091-1104.

Tierney *et al.* (1987), Planta 172: 356.

Tobkes *et al.* (1985), Biochem. 24:1915-1920.

Uchidaz, *et al.* (1980), in: Introduction of Macromolecules Into Viable Mammalian Cells, (Baserga *et al.* ,eds.) Wistar Symposium Series, Vol. 1, A. R. Liss Inc., NY, pp. 169-185.

Uchimiya, *et al.* (1982), in: Proc. 5th Intl. Cong. Plant Tissue and Cell Culture, (Fujiwara, ed.), Jap. Assoc. for Plant Tissue Culture, Tokyo, 507.

Vain, P.*et al.* (1996), Plant Cell Reports 15:489-494.

Vain *et al.* (1993), Plant Cell Rep. 12: 84.

Van Parijis *et al.* (1991), Planta, 183:258.

Vigers *et al.* (1992), Plant Sci., 83:155.

Viret, J.-F. *et al.* (1994), Proc. Nat Acad. Sci. 91:8577-8581.

Walbot *et al.* (1991), ISPMB Third International Congress, Tucson, AZ. Abstract No. 30.

White *et al.* (1990), Nucl. Acids. Res. 18: 1062.

Wigler, *et al.* (1979), Cell, 16:77.

Wilmink *et al.* (1995), Plant Molecular Biology 28:949-955.

Woloscuk *et al.* (1991), The Plant Cell, 3:619-628.

Xu et al (1994), Plant Physiol. 106:459-467.

Yamamoto *et al.* (1991), Plant Cell, 3(4):371-382.

Yanisch-Perron *et al.*(1985), Gene 33:103-119.

Zasloff (1987), PNAS USA, 84:5449-5453.

Zoller *et al.* (1984), DNA, 3:479.